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A Morphological and Histochemical Study of the Petromyzon Marinus Heart

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A MORPHOLOGICAL AND HISTOCHEMICAL STUDY OF
THE PETROMYZON MARINUS HEART

by

Theodore M. Beringer

A Dissertation Submitted to the Faculty of the
Graduate School of Loyola University
in Partial Fulfillment of the
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BIOGRAPHY

Theodore Michael Beringer was born May 1, 1944, in Denver, Colorado.

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I would also like to point out that my association with Dr. Harry Wang during the initial stages of my graduate education has left me with a deep respect both for him and the manner with which he applied himself to scientific investigation.

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ABSTRACT

This study has resulted in the description of the ultrastructural cellular organization in the young adult Petromyzon marinus heart, the distribution of its cardiac nerves, and the ultrastructural localization of acetylcholinesterase activity.

Cardiac innervation was studied using silver impregnation(Bodian, 1936, 1937), the thiocholine method for cholinesterase(Karnovsky & Roots, 1964), and electron microscopy. All three of the above methods revealed the presence of nerves within the sinus venosus but could not demonstrate the innervation of either atrium or ventricle. Since the brown sinus fibers resulting from acetylthiocholine incubation(3.5 hrs., 13°C) could not be observed after preincubation in 10^{-5} M BW284C51(Wellcome), a specific inhibitor of acetylcholinesterase, it is concluded that the sinus contains cholinergic fibers.

Electron microscopy showed the presence of non-myelinated axons within the sinus walls. At points of neuromuscular association, the axon protrudes from an incomplete Schwann cell investment and approaches within 400-500Å of an adjacent myocardial sarcolemma with only the basal laminae intervening between the two. No basal lamina is present in gaps less than 200Å. At these points of neuromuscular association, profuse electron lucent vesicles(450-500Å) are found within the axon crowded against the membrane nearest the myocardial cell. The electron lucent center of the vesicles did not vary with either of three fixative procedures: glutaraldehyde & OsO₄; glutaraldehyde, potassium dichromate(2.5%, pH 7.0), & OsO₄; and OsO₄ perfusion. Chromaffin cells were never observed to be innervated.

The application of an ultrastructural technique for acetylcholinesterase

(Karnovsky, 1964) revealed the presence of an electron dense product at two sites after acetylthiocholine incubation: the myofilaments near the A-I junction and subsarcolemmal segments of sarcoplasmic reticulum near the A-I junction. The filament reaction could be prevented by preincubation of the tissue in 0.1M N-ethylmaleimide(NEM), a sulfhydryl group blocker, whereas the reaction of the sarcoplasmic reticulum was not sensitive to NEM. The reaction of the sarcoplasmic reticulum could be prevented by preincubation in 10^{-5} M BW284C51, an inhibitor of acetylcholinesterase, and by eserine sulfate but not by tetraisopropylpyrophosphoramidate(10^{-5} M), an inhibitor of nonspecific cholinesterase.

I. INTRODUCTION

This dissertation is a study of the cardiac innervation in the young adult Petromyzon marinus (Lake Michigan lamprey). It is concerned with the histological distribution of cardiac nerve as revealed by morphological and histochemical means. It proceeds on the basis of this information to characterize the ultrastructural relationship between nerve and muscle with electron microscopy.

A. CARDIAC INNERVATION

Since cyclostomes occupy the oldest position in vertebrate phylogeny, the lamprey (Petromyzon marinus) is a logical subject for investigation of primitive cardiac innervation. Although it is believed that the lamprey heart is innervated by fibers originating in the vagus (Augustinsson et al., 1956), knowledge of intracardiac innervation derived from physiological and histological data is inconclusive. Furthermore, there is no ultrastructural evidence for the presence of nerve in cyclostome hearts.

In addition, fluorescence microscopy has failed to demonstrate the presence of adrenergic fibers (Falck et al., 1968) and most cyclostomes display a remarkable insensitivity to catecholamines (Augustinsson et al., 1956; Bloom et al., 1961; Ostlund, 1954). On the basis of this information and the fact that acetylcholine mimics vagal stimulation, lamprey cardiac innervation has been considered to be purely cholinergic (Burnstock, 1969).

It had been observed in the lamprey that stimulation of the median jugular vein at its junction with the pericardial cavity caused intense cardiac acceleration followed by bradycardia (Augustinsson et al., 1956); further, that acetylcholine exerted a pronounced cardiostimulatory effect on all lamprey hearts, including Petromyzon marinus (Jensen, 1969). These responses are contrary to normal cholinergic responses in mammalian cardiac tissue unless pretreatment with atropine is used to block the muscarinic or inhibitory response to acetylcholine.

As with acetylcholine, nicotine produces positive chronotropic effects in the isolated Petromyzon heart, suggesting that the cholinergic receptors associated with the lamprey myocardial cells are purely nicotinic (Augustinsson et al., 1956; Falck et al., 1966). Whether this nicotinic response is solely a cholinergic mechanism or an adrenergic action mediated via cholinergic nerves is of importance in order to understand the role of cholinergic dependent adrenergic mechanisms which may be operative within the heart (Burn and Rand, 1959; Koelle, 1961). However, a knowledge of morphological aspects of cardiac innervation remains a crucial prerequisite before such theories can be completely elucidated in any given biological system.

For these reasons it was decided to determine the extent of the cholinergic innervation utilizing a light microscopic technique for the demonstration of the enzyme cholinesterase (Karnovsky and Roots, 1964). The results of this investigation and those of silver impregnation (Bodian, 1936) were used to direct an electron microscopic search which has resulted in new information concerning the distribution and characterization of the nervous

components of the Petromyzon marinus heart.

B. MYOCARDIAL CELL CHOLINESTERASE

Biochemical data has shown the existence of cholinesterase activity within the lamprey heart (Augustinsson et al., 1956); however, such information does not indicate the morphological distribution of enzyme activity. Therefore, it was considered of interest to demonstrate the ultrastructural localization of cholinesterase activity within the myocardial cells due to the important role acetylcholinesterase may have in the poorly understood cholinergic mechanism of cardiac muscle.

II. LITERATURE REVIEW

The cyclostomes include two classes: the Petromyzonidae and Myxinoids. Petromyzon marinus or sea lamprey belongs to the former class and possesses a three-chambered heart suspended within a cartilaginous pericardium. Returning venous blood enters a small thin-walled tubular sinus venosus which opens into a somewhat more muscular atrium. From here blood is sent through a short extension of the atrium to the ventricle, the largest chamber of the heart. Blood is expelled from the ventricle through a short conus which is equipped with a pair of pocket valves. Valves are also present at the sinoatrial and atrioventricular junctions. Often small ligaments are present riveting the lateral part of the ventricle and sometimes the atrium to the internal aspect of the pericardium. As is common for cyclostomes, Petromyzon marinus lacks a coronary circulation.

A. INNERVATION OF THE CYCLOSTOME HEART

The results of the earliest investigations of cardiac innervation in cyclostomes show a marked difference between lampreys and myxinoids. It was thought initially that the lamprey heart received contributions from the vagus nerve (Young, 1962) and that the myxinoid heart was aneural (Jensen, 1961; Young, 1962).

Ideas opposing the role of cardiac regulation by extrinsic nerves in myxinoids were based on a lack of response after stimulation of the vagus,

medulla, and roots of the cranial nerves (Greene, 1902; Carlson, 1904).

Further evidence supporting the aneural nature of the myxinoid heart originated from the failure of the hagfish to react to large doses of acetylcholine (Augustinsson et al., 1956; Jensen, 1958, 1961) or catecholamines (Chapman et al., 1963; Ostlund, 1954). Ultrastructure surveys of myxine have also failed to reveal nervous components in these hearts (Hoffmeister, 1961; Leak, 1969).

On the contrary, positive indications that the lamprey heart was innervated came from methylene blue staining of nerve fibers in the sinus venosus leading to the auricle in the species Ichthyomyzon castaneus and I. concolor (Carlson, 1906). It was shown by the same investigator that stimulation of the medulla exerted its usual influence on the heart as long as the sinus venosus was intact with the atrium.

However, some evidence resulting from silver impregnation suggested that "myelinated nerve trunks" and both large and small "ganglion cells" which were believed to be vagal did indeed exist in close proximity to the ventricle of the hagfish, Eptatretus stouti (Hirsch et al., 1964). The same investigator identified another system of large ganglion cells associated with granular argyrophilic fibrils distributed along the outer edge of the aorta and extending into the ventricle.

Nevertheless, interpretation of these argyrophilic structures as ganglion cells was clouded by the demonstration that similar cells giving a chromaffin reaction (Johnels and Palmgren, 1960) after fixation in a chromate-bichromate mixture were also argyrophilic (Bloom et al., 1961). Coordinated electron microscopic observations demonstrated that these cells contained

copious membrane-bound granules. Once these granular vesicles were isolated by ultracentrifugation, treatment with reserpine caused the release of adrenaline and noradrenaline into the supernatant (Bloom et al., 1961; Ostlund et al., 1960).

Subsequent chromatographic separation of these catecholamines indicated that adrenaline was the predominant catecholamine in the lamprey heart whereas noradrenaline was found in higher concentrations in the hagfish (Bloom et al., 1961; Ostlund et al., 1960).

Following these observations, a highly specific method for the histological localization of monoamines (Falck and Owman, 1965) showed that the primary catecholamines were confined to the chromaffin cells and that the lamprey heart lacked an adrenergic innervation (Falck et al., 1966).

After silver impregnation according to the protargol method of Bodian (1936, 1937), small nerve branches could be observed in the sinus venosus of hearts from Petromyzon fluviatilis and P. planeri (Augustinsson et al., 1956). Although the investigator could not succeed in stimulating this nerve, the histological presence of cardio-regulative nerves was confirmed by stimulation of either the medulla oblongata near the floor of the fourth ventricle or the median jugular vein which joins the sinus. Stimulation in both cases caused cardioacceleration at low frequencies whereas high frequencies resulted in acceleration followed by bradycardia.

Since acetylcholine mimics vagal stimulation, i.e. it produces a positive chronotropic and negative inotropic response in all lampreys tested (Augustinsson et al., 1956; Falck et al., 1966; Ottori, 1953), including Petromyzon marinus (Jensen, 1957, 1969), vagal innervation is considered to

be cholinergic (Burnstock, 1969). The positive chronotropic response due to acetylcholine led Augustinsson's group (1956) to speculate that acetylcholine acts by stimulating the ganglion cells in the heart, i.e. the cells now known to contain catecholamines. However, there is no evidence to confirm that preganglionic vagal fibers form synapses with intramural ganglion cells (Burnstock, 1969).

B. PHARMACOLOGY OF THE PETROMYZON HEART

Adrenaline and noradrenaline added to the perfusion fluid of Petromyzon fluviatilis hearts caused a slight initial depression followed by a slowly developing weak positive inotropic and occasionally chronotropic action (Augustinsson et al., 1956). Another investigator found that norepinephrine caused the same weak results in perfused Petromyzon hearts but that some animals were refractive to norepinephrine administration. However, in the latter study all the positive responses to catecholamines were readily blocked by Nethalide, a beta-receptor blocking agent (Falck et al., 1966). Other studies indicated only cardiac insensitivity to exogenous catecholamines in the lamprey (Bloom et al., 1961; Ostlund, 1954).

In contradistinction to catecholamines, Petromyzon hearts were highly sensitive to acetylcholine; producing positive chronotropic and negative inotropic responses followed by a period of slowing. Perfusion of the heart with hexamethonium reduced the sensitivity to acetylcholine by a factor of a thousand (Augustinsson et al., 1956; Falck et al., 1966). The responses to acetylcholine were not influenced by atropine or muscarine. The effect of

nicotine was identical to that of acetylcholine but weaker and could be blocked by hexamethonium. The pharmacological effects of both acetylcholine and nicotine were blocked by tubocurare.

Perfusion with eserine salicylate (10^{-6} M) had a slight positive inotropic effect but higher concentrations (10^{-4}) retarded the heart rhythm. Eserine did not enhance the effect of acetylcholine and in high concentrations rendered the heart insensitive to acetylcholine (Augustinsson et al., 1956).

C. ULTRASTRUCTURE OF CARDIAC INNERVATION IN GENERAL

The rather gross aspects of vertebrate heart innervation have been well known from dissection and serial examination of histological preparations for quite some time (Berkely, 1894a, 1894b; Woolard, 1926). Using Cajal's silver impregnation technique on young cats and dogs, Nonidez (1939) was able to trace the parasympathetic and sympathetic cardiac nerves back to their respective origins at the vagus and sympathetic ganglia, respectively. Since that time a number of silver techniques have been utilized on a variety of vertebrate hearts to elucidate the apparatus of terminal innervation (Hirsch et al., 1962, 1963, 1964, 1965). With the development of histochemical techniques capable of detecting monoamines (Falck and Owman, 1965) and cholinesterase activity (Karnovsky and Roots, 1964a; Koelle and Friedenwald, 1949) investigators were able to plot the separate distributions of adrenergic and cholinergic intracardiac nerve fibers, a distinguishing feature not afforded with previously used histological stains.

Along with those developments, electron microscopy was used to clarify

the nature of neuromuscular relationships in cardiac muscle, specifically with regard to nerve terminations on myocardial cells. Electron micrographs of individual axons winding among myocardial cells revealed the presence of one or more axons ensheathed in Schwann cells along with axons lacking Schwann cell investments, but no intimate relationships with the myocardial cell membrane could be demonstrated in these early studies (Fawcett and Selby, 1958; Grimely and Edwards, 1960; Hadek and Talso, 1967; Kish, 1960, 1961; Napolitano, 1964, 1965; Novi, 1968; Price, 1959; Rybak, 1966; Viragh and Porte, 1961). It was fairly obvious from these investigations that no discrete motor end-plate existed as in skeletal muscle innervation (Birks, 1960).

However, in the auricle of the eel, nerve processes containing abundant vesicular profiles and mitochondria were observed to pass within 300A of the myocardial sarcolemma. Similar and smaller distances between nerve and muscle were observed in the auricle of the snail heart (Baxter and Nisbet, 1963), the ventricle of the frog (Thaemert, 1966), the ventricle of the mouse (Thaemert, 1969), the atrioventricular node of the mouse (Thaemert, 1970) and the sinoauricular junction of the trout heart (Yamauchi and Burnstock, 1968).

From adjacent serial thin sections of A-V nodal tissue, montages and three dimensional illustrations could be constructed which more accurately charted the course of vesiculated nerve varicosities among muscle cells in the mouse heart. Perhaps more important was the demonstration of nerve contiguities with the sarcolemma which were no more than 150A apart. Vesiculated nerve processes were shown to reside within sarcolemma-lined tunnels or culs-

de-sac inside nodal cells and within deep grooves on the surfaces of nodal cells. The majority of these neuromuscular contiguities were measured to be 20 μ or less (Thaemert, 1970). Similar "intracellular" profiles of vesiculated nerve processes were seen in ultrastructure studies of Yamamuchi and Burnstock (1968) in the sinoauricular junction of the trout heart and in the interventricular septum of the guinea pig heart (Hirano and Ogawa, 1967).

The demonstration of cardiac nerves forming close apposition with myocardial cell membranes was an essential one because of certain theoretical and calculated limits placed on the distance at which neuromuscular transmission is effective derived from ultrastructure studies of the motor endplate (Eccles and Jasper, 1957) and smooth muscle innervation (Merillees, 1968). Merillees had suggested that 1000A was the maximum effective distance that a neurotransmitter could be released to effect the sarcolemma of smooth muscle. From neuromuscular relationships established in the heart, Thaemert (1970) suggested that a neuromuscular contiguity may be defined to exist when the "space or synaptic cleft between the vesiculated nerve process and the surface of a nodal cell is diminished to a point where it is devoid of a basal lamina" (Thaemert, p. 242, 1970).

Electron microscopic investigations of peripheral nerve have made it reasonably possible to distinguish between adrenergic and cholinergic nerves in the heart based on the vesicular content of their processes (for refs. cf. Geffen and Livett, 1971, and discussion within present text). Electron microscopic correlations implicate the granular, osmiophilic vesicle as a feature of adrenergic nerves (de Robertis and Iraldi, 1961) whereas clear lucent

vesicles appear to be characteristic of peripheral cholinergic axons (Richardson, 1964).

An electron microscopic autoradiographic study of the innervation of atrial myocardium in ^3H -norepinephrine injected rats correlated axons containing granulated vesicles with norepinephrine uptake (Wolf et al., 1963). This information was substantiated to a great degree when both ^3H -norepinephrine and ^3H -epinephrine were found to sediment as small particles in the microsomal fraction of rat heart homogenates (Potter and Axelrod, 1962).

D. ELUCIDATION OF THE ROLE OF ACETYLCHOLINESTERASE AT THE NEUROMUSCULAR JUNCTION

Ironically, the intense depressor effects of acetylcholine on the heart had been described (Hunt and Taveau, 1906) before it was identified as the active principle of ergot responsible for cardiac inhibition (Ewins, 1914). After studying the effects of various choline esters including acetylcholine, Dale (1914) defined two distinct types of action: an inhibitory or "muscarinic" action blocked by atropine and a stimulatory or "nicotinic" action paralyzed by excess nicotine. Loewi (1921) showed that a perfusate recovered from the frog heart after vagal stimulation could elicit negative inotropic and chronotropic responses in a recipient frog heart, thus offering the first evidence for release of a substance from nerve that mimicked the effects of nerve stimulation itself.

In 1926 Loewi and Navratil studied the similarity between acetylcholine and vagal extract ("Vagusstoff") on the heart and demonstrated the presence of an enzyme in the cardiac extract which destroyed acetylcholine.

They also showed that non-active vagal extracts gave the same activity as acetylcholine if re-acetylated. These authors also showed that physostigmine potentiated the effects of both acetylcholine and "Vagusstoff" (Loewi and Navratil, 1926). Later it would be shown that the action of eserine was to prevent the enzymatic destruction of acetylcholine.

Dale, Feldberg, and Vogt (1936) were able to show the appearance of acetylcholine in the venous fluid of perfused muscle after denervation. Furthermore, it was shown that transmission of excitation from nerve to the perfused muscle could be prevented by curare but that stimulation of the motor nerve fibers still caused the usual release of acetylcholine.

After stimulation of the nerve to the sartorius in a curarized frog, Fatt and Katz (1950) located an endplate potential across the muscle membrane at the neuromuscular junction and showed that this endplate potential could be reduced by lowering external sodium concentration. Depolarization of the endplate could also be initiated with the application of acetylcholine to the endplate (Fatt, 1950). The rate and amplitude of this depolarization could be enhanced with the addition of prostigmine, an effect similar to that described on whole muscle after a moderate dose of another cholinesterase inhibitor, eserine (Eccles, Katz, and Kuffler, 1942). The work of Brown, Dale, and Feldberg (1936) led them to believe that acetylcholine is hydrolyzed during the refractory period in order to enable the tissue to return to its original excitable condition.

Although there was some speculation that enough acetylcholinesterase (AChE) existed at the myoneural junction to efficiently hydrolyze the acetyl-

choline during the refractory period, Marnay and Nachmansohn (1938) compared the ability of homogenates of the nerve-free pelvic end with that of the homogenates of the innervated area of frog sartorius to hydrolyze acetylcholine and concluded that there was apparently enough enzyme to destroy the amount of acetylcholine liberated during synaptic transmission. Additionally it could be shown that diffusion of the transmitter may facilitate termination of acetylcholine effects (Eccles, Katz, and Kuffler, 1942).

With the development of the acetylthiocholine technique for localizing cholinesterase (ChE) activity, it was shown that ChE could be identified at motor endplates of rat intercostal muscle with the light microscope (Koelle and Friedenwald, 1949).

Using a copious array of esterase inhibitors, Denz (1953) concluded that the main enzyme in the synaptic trough of the myoneural junction was AChE with a lesser amount of non-specific cholinesterase. Barnett (1962) demonstrated ChE activity ultrastructurally on the plasma membrane of the muscle covering the junctional folds, primary and secondary synaptic clefts, parts of the muscle membrane covering the axon terminal, and on vesicular structures in the terminal axoplasm. The activity everywhere was blocked by eserine (10^{-4} M) or diisopropylfluorophosphate (10^{-5} M).

Davis and Koelle (1965) used gold thiocholine substrates and a specific control inhibitor of AChE, "BW284C51" (Burgen, 1949; Austin and Berry, 1953a, 1953b; Bayliss and Todrick, 1956), to demonstrate AChE on the surface of the postjunctional membrane and lesser activity at the axoplasmic membrane terminal but disclaimed staining in the junctional cleft, synaptic vesicles,

or mitochondria of the terminal axon.

Eranko and Teravainen (1967) demonstrated AChE in the peripheral complex of synaptic folds of the myoneural junction in rat skeletal muscle and a non-specific ChE in the teloglia cells.

Later it was possible to calculate the number of AChE sites at the motor endplates of the sternomastoid and diaphragm by beta track autoradiography using ^{32}P -DFP to bind the active site of AChE (Rogers et al., 1969). The accuracy of the quantification was corroborated by liquid scintillation counting.

It has been mentioned that ChE activity had been demonstrated long ago in the frog heart (Loewi and Navratil, 1926). Further extensions of this work on cardiac homogenates and filtrates could show ChE activity in various portions of vertebrate hearts (Mommaerts, 1953).

Antopol (1939) used a Warburg procedure to demonstrate a much higher concentration of ChE in the auricle than in the ventricle of the rabbit heart. This observation was duplicated and extended to other vertebrate heart homogenates (Girardier et al., 1960) by using the ChE inhibitor eserine with acetylcholine and tributyrine as substrates to show that enzymatic hydrolysis of acetylcholine is due predominantly to AChE (Bulbring et al., 1954; Girardier et al., 1960).

Employment of light microscopic techniques for localization of ChE has shown that enzyme activity was confined to nerve trunks of various diameters which were numerous in all parts of the conducting system of the beef heart and to ganglion cells which occurred most frequently in the sinoauricular

and atrioventricular nodes. Purkinje fibers appeared to be devoid of specific ChE activity (Kamijo and Koelle, 1955).

In a number of vertebrate hearts, histochemical distribution of ChE was localized to ganglion cells and in postganglionic fibers and enveloping nodal fibers. The investigators did not observe enzymatic activity inside or on the surface of myocardial cells, however (Mohr and Gerebetzoff, 1954). Extensions of this work to other vertebrate hearts indicated that nodal tissue is cholinergically innervated (Dumont and Drouin, 1954; Dumont, 1954).

Staining for AChE after cervical vagotomy and transection of cervical cardiac nerves (Navaratnam, 1968) showed a concomitant loss of AChE from cells of the dorsal motor nucleus of the vagus and depletion of the presynaptic terminals within cardiac ganglia. Additional studies using histochemical stains for AChE and a fluorescence histochemical method for monoamines before and after cardiac denervation in cats and dogs indicated that the residual cholinergic innervation of these hearts and lack of fluorescence after denervation was probably due to the existence of intramural postganglionic parasympathetic nerves (Norvell, 1970; Jacobowitz et al., 1967). Identical conclusions were arrived at by a number of other investigators using silver impregnation techniques after bilateral cervical vagotomy, bilateral thoracic sympathectomy, or treatment with reserpine (for refs. cf. Cooper, 1965).

It might have been considered then by the results of these classical experiments, that all cholinesterase in the heart was due to that found in ganglion cells and other nervous components of the heart, were it not for the application of electron microscopic techniques for cholinesterase to the

myocardial cells themselves. Cardiac muscle of rabbit embryos showed the presence of acetylcholinesterase in the abundant randomly distributed rough endoplasmic reticulum of the myoblast and occasionally in the nuclear envelope, Golgi complex, and subsarcolemmal cisternae (Hagopian et al., 1969). These sites provided evidence that the myocardial cells themselves are capable of acetylcholinesterase synthesis. Furthermore, it was demonstrated that myocardial cell cholinesterase activity appeared before the embryonic rabbit heart became innervated. All previous histochemical investigations of muscle cholinesterase revealed a close association of nerve terminal and muscle endplate cholinesterase.

Karnovsky (1964b) applied his technique for cholinesterase to adult rat heart and detected enzyme reaction product in the A-band near the A-I junction and on or near the inner surface membranes of the longitudinal elements of sarcoplasmic reticulum. Activity was greatest with butyrylthiocholine and somewhat diminished with acetylthiocholine indicating that the enzyme was that of a non-specific cholinesterase. All activity was inhibited by eserine and DFP(10^{-5} M). The activity of the A-band was considered to be due to myosincholinesterase (Kover and Kovacs, 1957; Kover et al., 1957; Marshal et al., 1959; Varga et al., 1954, 1955) and could be blocked with eserine. At this time the function of intracellular myocardial cell cholinesterase is unknown.

Cholinesterase is present in the lamprey heart too. The Warburg technique was employed (Augustinsson et al., 1948) to determine cholinesterase activity of Petromyzon fluviatilis hearts on acetylcholine (ACh), acetyl-B-

methylcholine over that of butyrylcholine. This conclusion was supported by the finding that the enzyme activity of the crude extracts was not increased by high concentrations of acetylcholine suggesting suppression of enzyme activity by high substrate concentration which is characteristic for acetylcholinesterase. Cholinesterase activity of whole heart homogenates was found to be 24.6 μ moles of acetylcholine hydrolyzed in 30 minutes by 1 gram of tissue. For the auricle and ventricle, cholinesterase activity was calculated to be 10.5 and 11.2 μ moles ACh/30 minutes/1 gram respectively. Nevertheless no histochemical or ultrastructural sites of cholinesterase activity have been described for the lamprey heart.

In addition to the well documented enzymatic property responsible for hydrolysis of acetylcholine, recently accumulated evidence has led certain investigators to attribute a cholinoreceptive function to AChE suggesting that the cholinoreceptor (ChR) is an intrinsic part of the AChE molecule. In attempting to identify the ChE, most work has depended upon the binding of various cholinergic ligands which are assumed to attach to the ChR (e.g. atropine, tubocurine, and eserine) as a prerequisite for receptor candidates. The results of this kind of work on subcellular fractions or extracts of nerve and muscle preparations have implicated a number of isolated products, including AChE and various phospholipids, as receptor candidates (for refs., cf. Ehrenpreis et al., 1969; de Robertis, 1971).

One theory has been proposed which equates the ChR with anionic centers of the AChE molecule (Zupancic, 1965, 1967). It is based largely on the similarity of equilibrium constants calculated from binding studies of

tubocurare to the ChR of leech dorsal muscle preparations (Krnjevic and Straughan, 1964) and of tubocurare to anionic centers of cholinesterases. The working hypothesis of this theory is that the anionic centers of AChE, as determined by Krupka (1964) for that enzyme exclusive of the esteratic site is identical to the cholinoreceptors.

Ehrenpreis (1969) has reasoned that one of the consequences of Zupanc's theorem is that the number of receptors should be numerically equivalent to the number of active sites of ChE, i.e. that the number of anionic drug-binding sites should be about the same quantity as the number of esteratic sites since the active center of the enzyme contains one anionic site and one esteratic site (Wilson, 1967). However, this has been difficult to demonstrate. In addition, new information proving that drugs such as atropine, which were initially presumed to bind exclusively to the ChR, bind to a multiplicity of materials isolated as possible receptors including AChE (Kato *et al.*, 1970; Kato and Yung, 1971), phospholipoproteins (Eldefrawi *et al.*, 1970), and proteolipids (de Robertis, 1971) make interpretation of binding experiments difficult if not inconclusive.

E. DEVELOPMENT OF HISTOCHEMICAL AND CYTOCHEMICAL TECHNIQUES FOR CHOLINESTERASE

Since the demonstration that esterases differ according to substrate preference and in their behavior toward inhibitors (Aldridge, 1954), numerous investigators have used substrate and inhibitor specificities as criteria for distinguishing between members of this class of enzymes.

For instance, diethyl-p-nitrophenylphosphate (E600) was originally

used to distinguish between two basic types of carboxylic esterases, types A and B (Aldridge, 1953a, 1953b, 1954). Type A-esterase is resistant to E600 and hydrolyses acetate esters at a higher rate than corresponding butyrates; type B-esterase is inhibited by low concentrations of E600 and hydrolyses butyrates at an equal or faster rate than acetates. Lipase type esterases are considered to belong to the B type category (Myers et al., 1955).

Later, a C-type esterase was found to hydrolyze simple carboxylic esters but at a lesser rate than that by A-esterases. It could be distinguished from the A-type by its decreased resistance to organophosphorous compounds and by its activation with p-chloromercuribenzoate (Bergmann, 1957, 1958).

All the esterases mentioned above were found to demonstrate resistance to eserine inhibition and could be distinguished from cholinesterases based on the latter's complete inhibition by low concentrations of eserine (Easson and Stedman, 1937; Richtor and Croft, 1942; Englehart and Loewi, 1930; Mendel and Rudney, 1943; Goldstein, 1944).

However, two classes of cholinesterases capable of hydrolyzing choline esters were shown to exist in animal tissue. In subsequent studies it was learned that RBC-ChE (Alles and Hawes, 1940) could be differentiated from serum cholinesterase (Stedman, Stedman, and Easson, 1932) by the former's ability to hydrolyze acetyl-B-methylcholine, a substrate not split by serum esterase (Glick, 1941). Richtor and Croft (1942) found that RBC-ChE exhibited high specificity for acetylcholine.

Brain-ChE was shown to demonstrate these same properties exhibited by

the RBC enzyme (Nachmansohn and Rothenberg, 1945) and neither enzyme hydrolyzed benzoylcholine, a substrate split by serum-ChE (Mendel, Mundel, and Rudney, 1943).

Biochemical data indicated that acetyl-B-methylcholine was a specific substrate for acetylcholinesterase and that benzoylcholine was rather specific for non-specific or "pseudo"-ChE which also split a variety of non-choline esters (Mendel et al., 1943; Mendel and Rudney, 1943). Consequently, it was shown that pseudo-ChE splits long chain choline esters (e.g. butyrylcholine) more rapidly than AChE does (Ord and Thompson, 1952).

Correlating data from all of the above investigators, Augustinsson and Nachmansohn (1949) distinguished acetylcholinesterase from all other cholinesterases which they referred to as nonspecific or pseudo-cholinesterase. The former has a high affinity to acetylcholine (i.e. a small Michaelis constant), a high turnover number, splits butyrylcholine at a low rate or not at all, and is inhibited by excess substrate. The latter splits cholinesters but when compared with acetylcholinesterase has a high Michaelis constant for acetylcholine, are not inhibited by high concentrations of acetylcholine, and their ability to hydrolyze cholinesters increases with the length of the acyl chain.

With the discovery that sulfur analogues of cholinesterase (namely, acetylthiocholine and butyrylthiocholine respectively) are hydrolyzed by AChE at a faster rate than acetylcholine (Koelle and Friedenwald, 1949) and pharmacological actions which are weaker than but similar to acetylcholine, these analogues were utilized as substrates for histochemical reactions (Koelle

and Friedenwald, 1949; Karnovsky and Roots, 1964) and ultrastructural cytochemistry (Karnovsky, 1964). They have also been incorporated into biochemical procedures as satisfactory substitutes for the natural substrate (Ellman et al., 1961; Tabachnik, 1956; Gal and Roth, 1957; Meyer and Wilbrandt, 1954). Although the substrate specificities of AChE and pseudo-ChE overlap slightly with regard to acetylthiocholine and butyrylthiocholine, these substrates are capable of distinguishing between AChE and BuChE (butyryl-cholinesterase or pseudo-cholinesterase) when employed with the appropriate anticholinesterase.

The ability of eserine sulfate to distinguish between cholinesterases and aliesterases by its inhibitory effect on the former as a group is well documented (Mendel and Rudney, 1943; Richtor and Croft, 1942) and has been shown to inhibit completely cholinesterase in light microscopic sections of rat heart (Karnovsky and Hug, 1963) and in rat heart homogenates (Girardier et al., 1960).

Secondly, tetraisopropylpyrophosphoramidate is a selective irreversible inhibitor of pseudocholinesterase and highly selective in discriminating between RBC-ChE and plasma-ChE (Aldridge, 1953; Austin and Berry, 1953).

Another anticholinesterase BW284C51, 1,5-bis (4-allyldimethylammoniumphenyl) pentane-3-one diiodide, is a selective competitive reversible inhibitor of AChE (Augustinsson, 1963; Fulton and Mogey, 1954; Burgen, 1949; Long, 1963). This anticholinesterase is also highly selective in discriminating between RBC and plasma-ChE. The results of BW284C51 inhibition can be compared with the inhibitory effect of tetraisopropylpyrophosphoramidate to distinguish AChE from pseudo-ChE respectively because of a high degree of specifi-

city exhibited by these anticholinesterases (Austin and Berry, 1953a, 1953b). BW284C51 has been shown to inhibit 97-98% of rat brain AChE and only 1-2% of the pseudo-ChE activity (Bayliss and Todrick, 1953).

III. MATERIALS AND METHODS

A. RESEARCH ANIMAL

The young adult lamprey (Petromyzon marinus) was utilized in all experiments. Specimens approximately six inches in length were acquired from the Hammond Bay Biological Station near Millersburg, Michigan and flown to Chicago where they were maintained in aerated water at 13-15°C and fed with yeast weekly.

B. PREPARATION FOR TRANSMISSION ELECTRON MICROSCOPY: GENERAL ULTRASTRUCTURE

Hearts from nine animals were prepared in the following manner: Before the heart was dissected free, most lampreys had been decapitated, although some were stunned by a blow to the skull and a few anesthetized with an aqueous solution of tricaine methane sulfonate (Chapman et al., 1963) at a concentration of 1:5000 by weight (Merck Index, 8th edition).

Pieces of tissue no larger than 1mm^3 were cut from all three chambers of the heart and immediately immersed in 3.125% aqueous glutaraldehyde solution (Sabatini et al., 1962, 1963) buffered to pH 7.2 with 0.15M phosphate buffer (Millonig, 1961) and left for one hour. After three one-hour washes in Millonig's phosphate buffer, the tissue was postfixed in phosphate buffered 1% OsO_4 for one hour (Millonig, 1961). Tissues were then dehydrated in increasing concentrations of chilled ethyl alcohol baths for ten minutes each, followed by two fifteen minute periods in propylene oxide.

All tissues were subsequently infiltrated with increasing proportions of Epon:propylene oxide mixtures followed by a twelve hour immersion in pure Epon 812 (Luft, 1961). Epon imbedded blocks of tissue were polymerized in a 60°C oven for 48 hours, then sectioned with glass knives (Latta and Hartmann, 1950) on a Porter and Blum MT1 or a Reichert OmU2 ultramicrotome. Sections were placed on copper grids coated with formvar or collodian or on bare grids and contrast enhanced with uranyl acetate (Swift and Rasch, 1958) and/or lead citrate (Reynolds, 1963). Sections were examined on an RCA EMU 3F-2 electron microscope at 50 KV.

C. TISSUE PREPARATION FOR SCANNING ELECTRON MICROSCOPY

The hearts from two animals were prepared for viewing with the scanning electron microscope. Tissue preparation for SEM was identical to those steps outlined above for TEM through dehydration with absolute alcohol but infiltration and imbedding were omitted. Instead the entire hearts were dried in an evacuated dessicator in the presence of CaSO_4 at room temperature. The ventricles were bisected and then mounted on aluminum chucks with Duco cement and coated with a 200-400Å layer of gold prior to observation with a Cambridge "Stereoscan Mark II A" scanning electron microscope.

D. SILVER IMPREGNATION OF PARAFFIN SECTIONS: BODIAN METHOD

Six animals were used for this procedure. Cross-sections through the entire width of the body large enough to include the intact heart were fixed in alcoholic Bouin's solution (Williams, 1962) for 48 hours. The hearts from

two other animals were first dissected free and then fixed in an identical manner.

Alcoholic Bouin:	ethanol, 80%	100 ml.
	neutral formalin	40 ml.
	glacial acetic acid	10 ml.
	picric acid	2 grams

Fixed tissue was washed, dehydrated in increasing concentrations of ethyl alcohol, cleared in xylene, infiltrated and imbedded in paraffin. Sections (12u) were mounted on albumen treated slides and processed according to the copper protargol method (Bodian, 1936, 1937) outlined below:

1. Removal of paraffin with xylene and hydrated through decreasing concentrations of ethyl alcohol to distilled water.
2. Sections placed in 1% protargol (silver albumose) with 5 grams of metallic copper per 100cc of solution for 12-24 hours at 37°C.
3. Wash in distilled water and reduce in hydroquinone bath (10 min.):
 - 1 gram hydroquinone
 - 5 gram sodium sulphite
 - 100 ml. distilled water.
4. Wash in distilled water and tone in a 1% gold chloride solution containing 3 drops of glacial acetic acid per 100 ml. of solution until sections are decolorized.
5. Wash in distilled water and place sections in 2% oxalic acid (5 minutes) until blue.

6. Remove residual silver salts in 5% sodium thiosulphate (10 minutes).
7. Wash, counterstain, and dehydrate in ethyl alcohol and mount in Harleco's synthetic resin.

E. SILVER IMPREGNATION OF PARAFFIN SECTIONS: HOLMES' METHOD

Two animals were used for this procedure. Paraffin sections (15u) of neutral formalin fixed tissue are processed according to Holmes' method (Holmes, 1943):

1. Place hydrated slides in 20% silver nitrate at room temperature and in the dark (2 hours).
2. Wash in distilled water and place in a volume of impregnating solution equal to 20 ml. per slide. Impregnate 24 hours at 37°C. Impregnating solution: Mix 55 ml. of boric acid buffer with 45 ml. of borax buffer and dilute to 494 ml. with distilled water. Add 1 ml. of 1% silver nitrate and 5 ml. of pyridine (10% in distilled water).

Boric acid buffer:	12.4 grams	boric acid
	1000 ml.	distilled water

Borax buffer:	19 grams	$\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$
	1000 ml.	distilled water

3. Place slides in reducing solution warmer than 25°C (2 minutes).

reducing solution:	hydroquinone	1 gram
	sodium sulphite	10 grams
	distilled water	100 ml.

4. Wash in running water and then distilled water.
5. Place in 2% oxalic acid (10 minutes).
6. Rinse in distilled water and transfer to 5% sodium thio-sulphate (5 minutes).
7. Wash in tap water (10 minutes), rinse in distilled water, dehydrate, and mount in Harleco's synthetic resin.

F. HISTOLOGICAL THIOCHOLINE METHOD FOR CHOLINESTERASE

Whole cross-sections through the body of the lamprey (7 animals) were cut at the level of the heart and fixed at 4°C for twelve hours in 10% formalin containing 1%CaCl₂ buffered with 0.075M phosphate buffer adjusted to pH 7.6 with NaOH. Tissue blocks were then washed in distilled water and frozen in an acetone-dry ice mixture and maintained in the frozen state. Serial sections (12u) were cut through the entire heart with a cryostat and mounted on gelatinized coverslips. These sections were then incubated in Karnovsky-Roots (1964) media containing either of two substrates: acetylthiocholine iodide or butyrylthiocholine iodide (Calbiochem, Los Angeles, California).

Karnovsky and Roots Incubation Medium: Acetylthiocholine or butyrylthiocholine iodide (5 mg.) was dissolved in 6.5 ml. of 0.1M sodium hydrogen maleate buffer (pH 6.0). The following aliquots of freshly prepared stock solutions are then added in sequence with agitation each time:

- 0.5 ml. 0.1 M sodium citrate
- 1.0 ml. 30mM CuSO₄
- 1.0 ml. distilled water
- 1.0 ml. 5mM potassium ferricyanide

0.1M sodium hydrogen maleate buffer, pH 6.0

26.9 ml. of 0.2M Na OH
50.0 ml. of 0.2M NaH maleate (see below)
plus distilled water to make a total of 200 ml.

0.2M NaH maleate

19.6 maleic anhydride
8 grams Na OH
plus distilled water to make 1000 ml. total

Incubation was carried out in glass Columbia jars at 13°C (3.5 hours). Control media contained one of two cholinesterase inhibitors [B.W.284C.51 or eserine sulfate; see section G] or lacked substrate. Coverslips with incubated sections were counterstained and mounted on slides with a glycerol-gelatine mixture and examined with the light microscope.

All sections were preincubated for 30 minutes in medium without substrate at room temperature before incubation in medium with substrate. Those sections to be incubated in media with substrate plus enzyme inhibitor were preincubated in media without substrate but including the inhibitor for thirty minutes at room temperature.

With each new animal treated according to the above procedure, the ratio of sections treated with the acetylthiocholine substrate to those treated as controls was increased so as to get a more complete histological picture of cholinergic distribution within each heart.

G. ULTRASTRUCTURAL THIOCHOLINE METHOD FOR CHOLINESTERASE

Tissue was prepared according to a modified technique of Karnovsky (1964). The modifications included elimination of sucrose from the incubation

medium and fixation with paraformaldehyde instead of with formalin (Karnovsky, 1970); personal communication). Ventricles were dissected free from stunned young adult lampreys and allowed to beat briefly in cold phosphate buffer to expel blood. The ventricles were then immersed in 4% paraformaldehyde buffered with 0.075M phosphate buffer adjusted to pH 7.6 for three hours at 4°C. After fixation, the tissue was stored at 4°C for 16 hours in 0.1M N-ethylmaleimide (Calbiochem, Los Angeles, California) in 0.1M phosphate buffer adjusted to pH 7.6 or in buffer alone. After this procedure, the tissue was placed in a polyethylene container and quick frozen in an acetone-dry ice mixture. Frozen sections were cut at a thickness of 60u and placed in various preincubation media, e.g. sections to be incubated ultimately in either of two substrates (acetylthiocholine or butyrylthiocholine) were preincubated in the medium without substrate; sections to be incubated in media with an enzyme inhibitor (anticholinesterase) were preincubated in media without substrate but including the inhibitor. Preincubation was done at room temperature for thirty minutes in clean test tubes. Final incubation in media with substrate was completed in test tubes containing media identical to those described in the section on light microscopic localization. However, sections were incubated for 30 minutes and the incubation temperature was reduced to 4°C. The final inhibitor concentrations were 10^{-5} M.

After incubation, the 60u sections were washed in maleate buffer for ten minutes and transferred by glass rods into 1% OsO_4 buffered with s-collidine to pH 7.2 (Bennett and Luft, 1959) where they were allowed to fix for thirty minutes. Sections were subsequently dehydrated with increasing concen-

trations of chilled ethyl alcohol baths for ten minutes each, immersed in propylene oxide for two ten minute periods, infiltrated, and imbedded in Epon 812 (Luft, 1961). Subsequently, sections were cut on a Reichert OmU2 ultramicrotome. Gold sections were picked up on uncoated copper grids. Contrast was enhanced by treating these sections with lead citrate (Reynolds, 1963) although some grids were examined without "staining" to evaluate the effect of lead citrate on the enzymatic reaction product which is copper ferrocyanide.

Sections from two animals were incubated in acetylthiocholine, butyrylthiocholine, or no substrate. Following this procedure, sections from two animals were pretreated with N-ethylmaleimide as detailed above followed by incubation in either or both substrates. In the last group, sections from three animals were pretreated with NEM followed by incubation with acetylthiocholine (AcThCh), AcThCh and B.W.284C.51, AcThCh and eserine sulfate, and AcThCh and tetraisopropylpyrophosphoramidate.

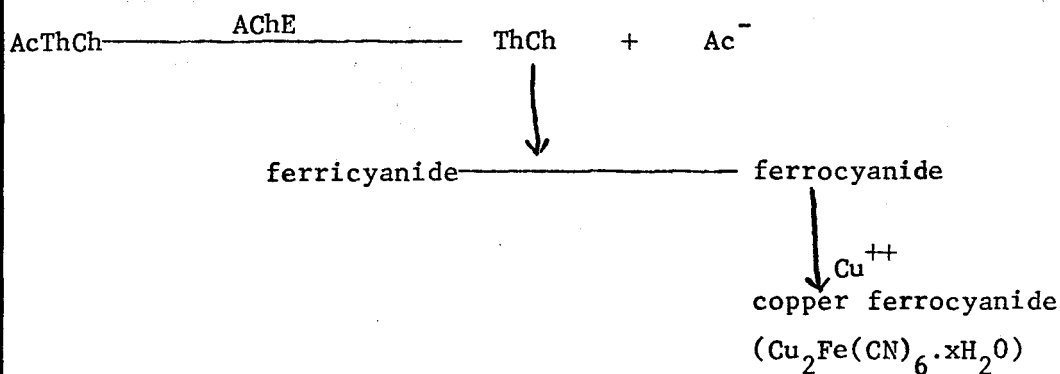
Inhibitors

1. B.W.284C.51 [1:5-bis-(4-trimethylammoniumphenyl) pentan-3-one dibromide] is a competitive reversible inhibitor of acetylcholinesterase (Austin and Berry, 1953; Burgen, 1949; Bayliss and Todrick, 1956). Wellcome Research Laboratory.
2. Eserine sulfate (Physostigmine·H₂SO₄) is a reversible inhibitor of cholinesterases as a group (Nachmansohn and Rothenberg, 1945; Richtor and Croft, 1942; Easson and Stedman, 1937; Mendel and Rudney, 1943). Calbiochem, Los Angeles, California.
3. Tetraisopropylpyrophosphoramidate is an irreversible inhibitor of non-specific cholinesterase (Austin and Berry, 1953;

Aldridge, 1953). . Sigma Chemical Company, St. Louis,
Missouri.

BASIS OF THE REACTION:

According to Karnovsky (1964b) thiocholine is produced by the enzymatic hydrolysis of either acetylthiocholine or butyrylthiocholine substrates. The liberated thiocholine preferentially reduces ferricyanide to ferrocyanide which reacts with Cu^{++} ions to form an insoluble precipitate of copper ferrocyanide. This reaction product appears as a brown deposit in histological preparations and as an electron-opaque deposit in electron micrographs. In the incubation medium, the Cu^{++} ions are bound to citrate, to prevent the spontaneous precipitation of copper ferrocyanide.



1% OsO_4 in 0.1M s-collidine buffer: This fixative was prepared by diluting an aqueous 2% OsO_4 solution with an equal volume of 0.2M s-collidine stock buffer (see below).

0.2M s-collidine buffer, pH 7.4 - 7.45: Dissolve 2.67 ml. of pure s-collidine in about 50 ml. of distilled water, adding 9 ml. of 1N HCl and diluting to 100 ml. with distilled water.

H. PREPARATION OF SINUS VENOSUS FOR ELECTRON MICROSCOPIC CHARACTERIZATION OF NERVOUS ELEMENTS:

Once the results of silver impregnation and histological ChE activity indicated that the sinus venosus was the sole innervated structure, fixation was designed to facilitate the structural identification and characterization of nervous elements in that region. Therefore, the sinus venosus was prepared by three fixative procedures:

1. The sinus venosus from three animals was dissected free under a dissecting microscope and fixed in phosphate buffered glutaraldehyde, rinsed in three changes of phosphate buffer, and postosmicated. The fixative solutions were identical to those used for the general ultrastructural investigation of the heart and described in more detail in the materials and methods on page 23.
2. The sinus venosus from two animals was dissected into view under a low power light microscope and severed at its entry into the pericardial cavity. Then the needle of a syringe containing Millonig's phosphate buffered osmium tetroxide (1%, pH 7.2) was inserted into the atrial cavity and its contents slowly ejected so as to perfuse the sinus venosus. The "reverse" flow with fixative was necessary in this perfusion procedure since the venous structures entering the sinus venosus are too small in diameter and too fragile to allow insertion of a needle for perfusion in the direction of blood flow.

3. Alternately, Bloom and Barrnett's modification (1966) of Wood and Barrnett's procedure (1964) for ultrastructural localization of norepinephrine in vesicles of autonomic nerve endings was employed. The sinus venosus from two animals was dissected free and fixed in 3.125% phosphate buffered glutaraldehyde for one hour, rinsed in phosphate buffer for fifteen minutes, incubated in 2.5% potassium dichromate in phosphate buffer at pH 7.0 for one hour, and then refixed in phosphate buffered 1%OsO₄ for 1 hour.

After following previously described imbedding procedures, a few sections were cut from the block containing an entire or nearly entire cross section of the sinus at a point most distant from its juncture with the atrium. These sections were placed on slot grids and examined in the electron microscope to determine the position of nerve fibers within the block. The block was then removed from the microtome and the face "cut down" to facilitate sectioning, being careful not to cut away the portion containing the nerves. The block was re-entered into the microtome and pale gold sections cut from the sinus in a direction towards the atrium. These sections were placed on slot grids or 200 mesh grids and examined with the electron microscope to characterize the relationship of axons within the sinus.

TABLE I. Number of Animals Used According to Technique

<u>Technique</u>	<u>Number of Animals</u>
Transmission Electron Microscopy (General Ultrastructure)	9
Scanning Electron Microscopy	2
Bodian Silver Impregnation	6
Holmes Silver Impregnation	2
Karnovsky Histological Method for Cholinesterase	7
Karnovsky Ultrastructural Method for Cholinesterase	7
Ultrastructure Preparation for Sinus Nerves	7
Total Number of Animals Used	40

IV. RESULTS

A. ULTRASTRUCTURAL ORGANIZATION

Intracardiac distribution of blood in the lamprey has been understood to pass to all regions of the cyclostome heart through a system of intertrabecular spaces (Leak, 1969) due to a known lack of coronary arteries. Although light microscopic observations seem to verify this trabecular arrangement of muscle tissue in the atrium, it is not a totally accurate picture of the cellular architecture of the sinus venosus which is really only a tube and of the ventricle whose organization includes a channel network.

It is apparent from light microscopic observations of the ventricle that in addition to the trabecular organization, there also exists a channel system which originates from the ventricular cavity. These channels ramify radially away from their origin at the ventricular cavity and bifurcate along their course, becoming smaller in luminal dimension along their paths. In histological preparations, these channels are larger nearer the ventricular cavity than distally where they are smaller and trabeculae more common (Fig.1).

Scanning electron microscopy reveals the presence of circular openings (10-20 μ in diameter) in the ventricular endocardium (Fig. 2). These openings into the ventricular lumen are construed as the origin of channels seen with light microscopy. It can be observed in Figure 2 that the endothelial cells lining the ventricular cavity dip into the channel lumen to become continuous with the endothelial lining of these "endocardial channels" (Beringer, 1970).

The trabeculae originate from the muscular walls of these channels, i.e., as the channel wall breaks up it generates a number of trabeculae which fan out and abut upon the epicardial epithelium (Fig. 1).

Transmission electron microscopy verifies that a single layer of endothelial cells lining these channels determine the thickness of their walls by their relative shapes and linear densities. In larger channels these endothelial cells are somewhat round and densely packed with the long axis of the nucleus parallel to the axis of the lumen. In channels of smaller luminal dimension, the endothelial cells are less frequent and flat. Often, the nucleus of the endothelial cells lining the smaller channels is imbedded perpendicularly in the channel wall sending out cytoplasmic extensions which line the lumen. Stretches of rough endoplasmic reticulum are scattered throughout these cytoplasmic extensions.

Nuclei of the endothelial cells surrounding the trabeculae are oriented parallel with the axis of the trabeculae. These endothelial cells are thickest at the nuclear bulge and then taper off, ensheathing the trabeculae in slender cytoplasmic extensions as thin as those lining the channel lumen. In all cases, the endothelial lining of channels and trabeculae are approximately a thousand angstroms in width except where the endothelial cell nucleus is present (Fig. 3).

There are at least three other cell types within the substance of these trabeculae and channel walls, namely: myocardial cells, chromaffin cells, and fibroblasts (Fig. 4).

A diffuse basal lamina is present surrounding the muscle cell (Fig. 5).

A basement lamina of similar dimension is also found around the chromaffin cells (Fig. 6) but is absent around fibroblasts (Fig. 4). Although, a basement membrane is evident subendothelially along the trabeculae and channels, it is more obvious beneath the epicardium because of its greater density at this location (Fig. 7).

Scattered bundles of collagen are occasionally found between the endothelial lining and myocardial cells (Fig. 4) but are most commonly lodged under the cardiac epithelium. These subepithelial collagen aggregates are more numerous in the sinus venosus than in the other two chambers of the heart (Fig. 7). Distribution of collagen fibrils is more scant in the myocardium proper so that only a small number of collagen fibrils are occasionally seen laced between myocardial cells (Fig. 4).

B. SILVER IMPREGNATION FOR CARDIAC NERVE

Silver impregnation was used to facilitate an electron microscopic search for nerve and to point up any discrepancy with the histological picture of cholinergic distribution provided by the results of the Karnovsky-Roots method for cholinesterase.

Silver impregnation was carried out on cross-sections of whole young adult lampreys in order to use known sites of innervation to indicate the effectiveness of the impregnation procedure. Two impregnation procedures were tried, the Bodian (1936, 1937) and Holmes' techniques (1943). However, the results of the Holmes' procedure were unsatisfactory in staining peripheral nerve to body musculature and motoneurons in the spinal cord of this animal.

On the other hand, the Bodian protargol method seemed satisfactory. Those sites which gave strong silver impregnation were the anterior roots of the spinal cord, the motoneuron cell bodies, and axons of the central, medial, the lateral grey matter of the spinal cord, as had been demonstrated previously in silver preparations of lamprey spinal cord (Schultz et al., 1956). The results of silver impregnation were also seen in the parietal muscle where nerve processes run between adjacent layers of these fibers (Peters and Mackay, 1961).

Large granular argyrophilic cells corresponding to the argyrophilic chromaffin cell described by Bloom et al. (1961) were distributed throughout the entire heart (Fig. 8, 9, and 10). The distribution of this cell type matched the ultrastructural distribution of cells containing osmiophilic granules, i.e., just under the endothelial lining of the endocardial channels and trabeculae.

The only chamber showing a linear or filamentous display of argyrophilia that could be interpreted as axons was the sinus venosus. In one animal impregnated for twelve hours, no cardiac nerves were discernible so impregnation times for the other five animals were lengthened to 24 hours. Of these five animals, the sinus venosus of two animals were heavily innervated and two only slightly innervated while one showed no signs of innervation. All nerves entered the sinus at the ventral aspect of the cartilagenous pericardium where the median jugular and hepatic veins join the sinus. The dorsal aspect of the pericardium is the point at which the duct of Cuvier formed by the two common cardinal veins joins the sinus and it gave no evidence of innervation.

Figures 8 and 9 illustrate argyrophilic fibrils in the wall of the sinus venosus near the sino-atrial junction. The fibrils do not appear to continue beyond the sinoatrial valves to enter the atrium. Numerous argyrophilic cells can be observed in the atrium with centrally placed nuclei. These cell types are the chromaffin cells which occur throughout the heart and a few can be seen in a tangential portion of the sinus wall also. They were shown by Bloom (1961) to contain catecholamines and their ultrastructural appearance is shown in Figure 6.

Note that the portion of the sinus entering the pictures from the right in Figures 8 and 9 is heavily innervated compared to the sinus portion entering the field from the left. The sinus on the right receives venous blood from the median jugular and hepatic veins. The sinus wall in the left of the field is continuous with the duct of Cuvier and lacks innervation.

Figure 10 shows the junction of the sinus with the atrium in another specimen. There are no argyrophilic fibrils at the sinoatrial junction although the area contains many silver staining chromaffin cells. This particular site is the most likely route that the sinus nerves have available for entry into the atrial musculature. Interpretation of this area is made difficult, however, by the fact that muscle cell nuclei, here as elsewhere in the heart, stain with silver. Furthermore, the pleomorphic chromaffin cells can appear similar to very thick nerve fibers because their long processes often appear in a section although their main cell bodies containing the nucleus may be outside that plane of section.

C. HISTOLOGICAL DISTRIBUTION OF CHOLINESTERASE

The brown reaction product deposited in the tissue after incubation in the acetylthiocholine medium of Karnovsky-Roots was deployed in areas rendered argyrophilic by the Bodian procedure exclusive of the chromaffin cells. Therefore, the cholinesterase technique supplied a point of distinction between cholinergic innervation and the general distribution of argyrophilic catecholamine-containing cells in the heart. Axons, motoneurons, and the anterior roots of the spinal cord stained heavily with the Karnovsky-Roots procedure for cholinesterase as well as the more peripheral nerves supplying the parietal musculature of the body wall. Preliminary work with butyrylthiocholine substrate gave negative results for such nervous structures in the heart.

The spinal cord and body muscle innervation were used as a reference for the effectiveness of the cholinesterase procedure and were useful for demonstrating the effect of anticholinesterases on the staining of nerve sections incubated with acetylthiocholine. Figures 11 through 14 show the reactions of these nervous structures to incubation in media with and without acetylthiocholine and with the substrate plus either of the two anticholinesterases employed. The pictures include approximately one-half of the spinal cord (which is flattened dorsoventrally in cyclostomes), a portion of the anterior root on one side plus an area of the highly innervated body wall musculature. The large spherical structure below the spinal cord is the notochord. Figure 11 is a section of the above area incubated in acetylthiocholine without inhibitors (anticholinesterases). Most axons of the cord are positively stained brown as in the anterior root and nervous elements running between

adjacent plates of parietal muscle in the wall. Figure 12 shows a section incubated in Karnovsky's medium without substrate and is devoid of this brown reaction product in these areas. The vertically arranged dark appearing area at the base of the body wall musculature includes a number of granular pigment cells. Once they are identified in the section incubated without substrate, they can be recognized more easily in the same area as Figure 11. Deposition of the brown reaction product has been completely prevented in Figure 13 which shows a section incubated in substrate along with B.W.284C.51(10^{-6} M), a specific inhibitor of acetylcholinesterase. Figure 14 is a section treated with acetylthiocholine and eserine sulfate (10^{-6} M), a group-specific inhibitor of cholinesterases in general. It was estimated to be about 90% effective in preventing cholinesterase activity. Some brown product can be visualized at the base of the internal row of parietal musculature and at similar sites between the muscle rows where the nerves are more dense before splitting up to innervate individual muscle plates.

Examination of serial sections through the entire heart region revealed brown staining fibrils within the sinus venosus only. No similarly appearing fibrils were observed in either the atrium or ventricle. Among the group of animals examined for cholinesterase activity, nerves could not be observed to advance as close to the atrium as were seen in two silver preparations. As with silver impregnated sections, cholinesterase positive fibrils were observed only in the ventral portion of the sinus. In order for the anticholinesterases to have any meaning, sections treated with the substrate and the various enzyme inhibitors had to come from the ventral portion of the sinus where the nerves were most heavily concentrated. Since the sinus is almost

horizontally oriented at this point, cross-sections through the animal yield cross sectional views of the sinus and only very short segments of nerves can be seen; and most are seen in cross-section only. Longer stretches of nerve can be observed farther into the sinus nearer the atrium where they are cut in longitudinal sections at that point. Figures 15 through 20 are cross-sections of the sinus in order to illustrate the effects of the anticholinesterases and Figure 20 is included to show the nerves in a horizontal course through the sinus.

Figure 15 is a section treated with acetylthiocholine only. This low power photomicrograph shows a portion of the unreactive atrium and ventricle and a cross-section through the sinus venosus which contains the brown reaction product. Also outside the pericardium can be seen some reactive striated muscle along with wisps of stained nerve. Figure 16 is a higher power photomicrography of the sinus area which was incubated in acetylthiocholine. At this magnification individual cholinesterase positive fibrils can be seen in the sinus, some cut in cross-section, others in short longitudinal sections. Figure 17 is an adjacent section which was incubated in the same media as the section in Figure 16 but without substrate. It is totally devoid of the brown reaction product. Figure 18 was incubated in acetylthiocholine and eserine sulfate (10^{-6} M) and although some slight reaction can be seen, it is essentially negative. Figure 19 was also incubated in acetylthiocholine but with B.W.284C.51(10^{-6} M), a specific inhibitor of acetylcholinesterase, and the sinus in this section is unreactive also. Figure 20 is a section of the sinus closer to the atrium and so is cut horizontally. It was incubated in acetylthiocholine without anticholinesterases and illustrates a number of cholines-

terase positive nerve fibers running among the myocardial cells which are stained light red with acid fuchsin. The presence of brown fibrils was observed in four of the seven animals studied.

D. CARDIAC NERVE ULTRASTRUCTURE

Electron microscopy substantiated the results of histological observations. Extensive examination of areas in the atria and ventricles of nine hearts failed to disclose ultrastructural evidence for the innervation of these chambers in the young adult Petromyzon marinus. However, as demonstrated with the two histological procedures, ultrastructure studies of the sinus venosus indicate that this chamber contains numerous axonal processes.

These sections through the sinus near its confluence with the median jugular and hepatic veins at the pericardial wall contain large bundles of axons ensheathed singly or in multiples within Schwann cells (Fig. 21, corresponds approximately to section of sinus shown in Figures 16 through 20). Small fascicles of sheathed axons are further surrounded by fibroblasts which make up part of the endoneurium. At this point in the sinus most distant from the atrium, the nerve bundles are largest and contain more axon profiles than in sinus areas nearer the atrium. These nerves course subepicardially at first just under the outer dense collagenous lining of the sinus. Thin sections cut closer to the atrium show nerve bundles which are diminished in reference to the number of axons each sheath contains. As sectioning proceeds through the sinus, the axons abandon their peripheral location in the wall to associate more with the deeper lying musculature (e.g., Fig. 24 and 26). This

closer neuromuscular relationship is represented by the appearance of nerve bundles with less extensive Schwann cell investments and a diminished number of collagen fibrils intervening between nerve and myocardial cells. Consequently, throughout most of the sinus, nerves are observed more commonly devoid of the fibroblasts comprising the endoneurium.

In the areas densely occupied by Schwann cell sheathed axons travelling together, each unit is surrounded by a basal lamina and separated by a loose layer of collagen, whether or not fibroblasts from the endoneurium are present (Fig. 22). Individual axons are never myelinated although the investing Schwann cell may make one (Fig. 24) or more loose revolutions around the axon (Fig. 23).

When Schwann cells contain more than one axon, the axons are separated from one another only by a 200\AA space (Fig. 21 and 22). No axons were observed completely devoid of Schwann cell associations.

Axoplasmic Contents: The axon profiles contain most elements commonly observed in peripheral nerve.

Mitochondria (.1 μ wide) are numerous and oriented longitudinally with the long axis of the nerve (Fig. 26).

Neurofilaments about 60\AA in diameter and of undetermined length are present (Fig. 25 and 26) along with microtubules (Fig. 23, 24, and 25). The microtubules are about 200\AA in diameter, run in the long direction of the axon (Fig. 23), and occasionally branch. It is estimated that neurofilaments are more common than microtubules in the larger axons whereas the ratio appears reversed in smaller axons. Neither of these components appear frequently near

clusters of synaptic vesicles.

Dense particles about 250\AA in diameter are randomly distributed throughout the axoplasm and are interpreted as glycogen.

Multilamellar (Fig. 24 and 25) and multivesicular bodies are occasionally observed in the axoplasm.

Two types of vesicles are present, granular (Fig. 21, 22, and 25) and agranular (Fig. 27, 28, 29, 30, and 32). Agranular vesicles measure about $400\text{--}550\text{\AA}$ in diameter, have clear centers and occur in large populations with other vesicles of the same type within the axon. The granulated vesicle is about 850\AA in diameter with a range between 700 and 1000\AA . It is not as spherical as the agranular type, often presenting a discoidal profile (Fig. 25). The granular vesicles are considerably less numerous than the clear type although they tend to congregate in small groups. The two different vesicles are seldom present in the same part of the axon. The clear vesicles occur at points where the axon and muscle cells come into close proximity with one another (Fig. 27 through 30); whereas, the larger vesicles with dense central cores are most commonly in parts of the axon not closely associated with muscle cells. These granular vesicles are, in fact, most often in nerve fascicles where more than one axon is travelling within its sheath as in Figures 21 and 25. In the immediate vicinity of the large granular vesicles are sometimes observed smaller granular vesicular cores which probably represent peripheral sections of the larger granular vesicles.

Nerve Contiguities: Close relationships could not be demonstrated between axons and chromaffin cells. Although axon bundles could be seen to pass

in the vicinity of chromaffin cells (Fig. 22), the axons were always completely ensheathed in a Schwann cell except when an exposed axon was facing a myocardial cell in such a vicinity (Fig. 31).

Conversely, axons were commonly observed in close proximity to myocardial cells. These occurrences were more common as sectioning proceeded along the sinus towards the atrium. On many occasions, the Schwann sheath is incompletely wrapped around the axon leaving the exposed axon facing the sarcolemma. The space between the axon and sarcolemma is only occupied by the basal lamina of both the nerve and muscle cell involved, and the space separating axolemma and sarcolemma is reduced to less than 500\AA (Fig. 24, 27, 28, 29 and 30). These kind of neuromuscular distances could be observed to be formed between one axon and more than one myocardial cell. For instance, Figures 27 and 28 represent different levels of section through the same axon. In Figure 27, the axon is exposed at a point where the Schwann cell is incompletely wrapped around it and protrudes from the rest of the sheath to within 500\AA of an adjacent myocardial cell. In Figure 28, the same close opposition established in Figure 27 is maintained but an additional one has been formed between the same axon and another muscle cell.

At least in one case, a neuromuscular contiguity was observed in which the distance between the exposed axon and the sarcolemma was less than 200\AA and no intervening basement membranes were present (Fig. 32).

E. MYOCARDIAL CELL CHOLINESTERASE

Incubation of frozen sections from two hearts in the Karnovsky-Roots

medium plus either AcThCh or BuThCh without the presence of anticholinesterases resulted in a non-specific deposition of electron-opaque material at a number of sites including the endothelial lining, the sarcolemma, and the myofilaments.

Since previous histochemical demonstrations of myocardial cell cholinesterase indicated that it was either a component of the sarcoplasmic reticulum (Hagopian et al., 1969; Karnovsky, 1964) or the result of myofilament activity, the additional reactive site at the sarcolemma prompted the interpretation that some randomly distributed tissue component was interfering with the histochemical reaction and causing a non-specific precipitation of copper ferrocyanide. Since the greatest weakness of the Karnovsky medium is the susceptibility of copper ferricyanide to reduction by free sulfhydryl groups in certain tissues, the above non-specific precipitation may be explainable on this basis. To test this possibility, two hearts were pretreated with N-ethylmaleimide (NEM, a sulfhydryl group blocker) after the aldehyde fixation and prior to sectioning and incubation in the medium with substrate. These sections were compared to sections from another heart incubated in substrate (AcThCh or BuThCh) without previous treatment with NEM.

Sections untreated with NEM and incubated in the AcThCh-medium resulted in the same widespread deposition of product on the endothelial lining, sarcolemma, and myofilaments (Fig. 33). This myofilament activity was most evident near the A-I junction and least evident closer to the H-band (Fig. 33). The deposition was as widespread with BuThCh-incubated tissue but to a lesser degree and in regard to myofilament activity was judged qualitatively to be diminished but similarly located (Fig. 34). The myofilaments normally present

the appearance shown in Figure 35 under preferred conditions of fixation (glutaraldehyde and postosmication without incubation). Figure 35 indicates excellent clarity of A- and I-bands and the resolution of three rows of cross bridges within the H-band. However, this kind of ultrastructural detail could not be retained after the long periods of incubation and pretreatment imposed upon the tissue for detection of cholinesterase activity.

After pretreatment with NEM incubation in AcThCh, all reactivity was eliminated from the endothelial lining, sarcolemma, and the myofilaments, revealing only a membrane active site subjacent to the sarcolemma in the area of the I-bands. This activity could be demonstrated very consistently at each I-band near the sarcolemma where a subsarcolemmal cistern could be found. Its demonstration even in sections unstained with lead citrate indicated that it was not a selective lead precipitate (Fig. 36).

On the other hand, incubation of tissue in BuThCh after pretreatment with NEM indicated virtually no activity whatsoever in the myocardial cell although rarely activity could be demonstrated as in Figure 37 which illustrates an absence of activity in subsarcolemmal membranes of one cell and slight reactivity in an adjacent cell. On the basis of substrate preference for AcThCh established by these experiments, tissue sections from three hearts were again pretreated with NEM and incubated in AcThCh-medium, or in AcThCh medium along with one of three anticholinesterases: B.W.284C.51, eserine sulfate, or tetraisopropylpyrophosphoramidate. ¶

Sections incubated in AcThCh produced an electron dense product in the subsarcolemmal cisternae near the I-band. The product seemed confined to a

short stretch of the membrane (Fig. 38) as before and extensions of the cisternae away from the I-band or the A-I junction did not react (Fig. 36 and 38). Subsarcolemmal cisternae (SSC) in the same area were free of electron dense material after incubation in medium lacking substrate (Fig. 39).

The effect of the anticholinesterases on the SSCs was indicative of specific AcChE activity. The reactivity was still present in tissue treated with tetraisopropylpyrophosphoramidate, an irreversible inhibitor of butyrylcholinesterase (Fig. 40). Figure 41 is an enlargement of Figure 40 and shows reactive focal points separated by about 150\AA along the membrane. On the other hand, eserine sulfate, a group specific inhibitor of cholinesterases, succeeded in eliminating the reaction of tissue incubated in AcThCh (Fig. 42) as did B.W.284C.51 (Fig. 43), a highly specific inhibitor of acetylcholinesterase.

Under preferred conditions of fixation, i.e., glutaraldehyde and postossification, in which tissues were not prepared for incubation, there were similar appearing membranes located subjacent to the sarcolemma (Fig. 44). These subsarcolemmal cisternae are most commonly observed in the area of the I-band and exhibit fixation superior to that generally observed in tissue prepared for the ultrastructural localization of cholinesterase. These SSCs are also more abundant than observed in frozen sections prepared for incubation because they are not subject to damage resulting from cryostat sectioning.

Figure 45 is an enlargement of an area in Figure 44. It indicates the two different structures assumed by these membranes which occur under the sarcolemma in the area of the I-band: one contains a central dense area and the other is without such a density. These two differently appearing membranes are

apparently continuous with one another as shown in Figure 46. The membrane reticulum with the central dense area is usually found 200Å under the sarcolemma whereas the membrane reticulum without a central density is usually not so consistently near the sarcolemma. These subsarcolemmal cisternae are apparently continuous with deeper lying reticulum which emerges toward the sarcolemma from around the myofilament mass (Fig. 47). Poor preservation of these membranes due to frozen sectioning and prolonged incubation times detracted from their appearance in sections incubated in thiocholine media. It was also difficult to distinguish sarcoplasmic reticulum with the central density from that without.

In cases where the reaction product is densest as in Figure 36 and 38, the reaction product obscures detail within these subsarcolemmal cisterns. It seems that the cholinesterase activity is present in both types of sarcoplasmic reticulum. Sections incubated in Karnovsky's acetylthiocholine medium without potassium ferricyanide or copper sulfate failed to react.

V. DISCUSSION

A. IMPLICATIONS OF THE DISTRIBUTION OF NERVE WITHIN THE PETROMYZON MARINUS HEART

The results of the three methods for detecting nerve in the Petromyzon marinus heart correlate so well with one another that it would be reasonable to assume that the sinus venosus is the only chamber which is significantly innervated in the young adult within three months beyond the ammocete stage.

The failure to find nerve in one of the ultrastructure preparations and in some of the silver and cholinesterase preparations of the sinus venosus could have either of two explanations. It is possible that the unpredictability of silver impregnation procedures in general may be responsible on the one hand. Also the cholinesterase may not have been preserved sufficiently to detect cardiac nerve. However, the fact that in these preparations other sites of innervation such as the body musculature displayed argyrophilia and cholinesterase reactivity suggests that this early stage in the developing young adult is critical for establishing the cardiac innervation. Because the age of these animals could not be exactly determined, the corresponding variability of cardiac innervation within the group of animals studied could be expected. Although the animals were approximately 5-6 inches in length and collected within a three month period from the termination of the larval stage, even a few days difference in age may have shown a wide range of results. For instance, the light micrographs illustrating nerve fibers extending almost to

the atrium but apparently ending just before the sino-atrial valves are from two of the largest animals. Smaller animals did not show nearly this extensive innervation.

The distribution of argyrophilic and cholinesterase containing fibers solely through ventral portion of the sinus leading to the atrium also suggests that in these early stages of innervation at least, nerves enter the heart from the opening in the cartilagenous pericardium which admits the terminal portion of the median jugular and ventral hepatic veins rather than from the dorsal entry of the duct of Cuvier. The presence of cardioacceleratory nerves there would also explain why stimulation of the median jugular vein elicits acceleration of the heart (Augustinsson et al., 1956). A further note supporting the view that the early stages of development in the young adult lamprey are critical for establishing cardiac innervation is the fact that stimulation of the median jugular vein in the ammocete or larval lamprey evokes no cardiac responses (Carlson, 1906).

Furthermore, the cardioacceleratory responses of the Petromyzon heart upon stimulation of the floor of the fourth ventricle near the dorsal motor nucleus of the vagus (Augustinsson et al., 1956) would seem to confirm the conclusion of early workers who dissected branches of the vagus towards the heart in the lamprey and decided the heart received nervous elements from the vagus (Julin, 1887; Ranson and Thompson, 1886). However, one conclusion concerning the innervation of the lamprey heart seems to lack proof of interpretation. For instance, the claims by Augustinsson et al., (1956) and Hirsch (1964) that the lamprey and hagfish heart contains ganglion cells based on

the results of silver impregnation are without much substance since it was demonstrated that the chromaffin cells of the cyclostome heart are argyrophilic and resemble ganglion cells morphologically (Bloom, 1961).

The acetylcholinesterase activity of sinus fibers described in this dissertation would seem to confirm the cholinergic nature of cardiac innervation in the Petromyzon. It had previously been estimated that the lamprey heart was innervated by cholinergic fibers based on the fact that acetylcholine mimicked vagal stimulation (Burnstock, 1969). Furthermore, the sole innervation of the Petromyzon heart may be cholinergic since fluorescence microscopy failed to demonstrate any adrenergic components (Dahl, unpublished results 1966; Falck et al., 1966).

Three fixative procedures were employed to characterize the sinus nerves in electron micrographs in order to make it conducive for any possibly existing adrenergic axons to display secretory vesicles with electron dense cores characteristic of adrenergic nerves. A large amount of evidence has accumulated over the last decade which is responsible for the generally accepted conclusion regarding peripheral nerve that under proper conditions of fixation, secretory vesicles containing electron opaque cores belong to adrenergic nerves as opposed to cholinergic axons which display secretory vesicles with clear centers. The idea that granular vesicles between 400-600A in diameter contain the adrenergic transmitter characteristic of adrenergic nerves was first suggested by de Robertis and de Iraldi (1961). Since then the following evidence has come to support the idea:

1. Small granular vesicles are indigenous to ultrastructural

- profiles of nerve in the dilator muscle of the rabbit iris which is predominantly innervated by adrenergic nerves (de Robertis, 1964).
2. The loss of norepinephrine in denervated organs is paralleled by the disappearance of the small granular vesicles (Potter et al., 1965; Van Orden et al., 1967).
 3. Exogenous tritiated norepinephrine can be localized by electron microscopic autoradiography to axons containing small granular vesicles (Devine and Simpson, 1968; Taxi and Droz, 1966; Wolfe et al., 1962) while preganglionic endings in the superior cervical ganglion, known as cholinergic, were never labelled with tritiated norepinephrine (Taxi, 1969).
 4. Microfluorimetric, electron microscopic, and biochemical investigations of the rat vas deferens show that catecholes and hydroxyindoles reduce OsO_4 resulting in an electron opaque product (Van Orden et al., 1966). Depletion of norepinephrine with alpha-methyl-meta-tyrosine, known to interfere with norepinephrine synthesis (Anden, 1964; Hess et al., 1961; Spector et al., 1965) also depleted the granular cores of sympathetic nerve vesicles which could be restored by incubation with norepinephrine. Reserpine depletes these granular cores paralleling a reduction in tissue norepinephrine and fluorescence (Van Orden et al., 1966, 1967).
 5. Small granular vesicles appear in synaptic vesicles of termi-

nals in regions of the brain known to have high amine content after fixation with potassium permanganate (Hokfelt, 1967, 1967).

6. Glutaraldehyde undergoes a first order reaction with norepinephrine to form a complex which is insoluble in solvents used in the preparation of tissue for electron microscopy (Hopwood and Coupland, 1965), although the chance of diffusion of this complex may increase with time on immersion in aqueous solvents (Geffen and Livett, 1971).

On the other hand, electron lucent vesicles have been implicated strongly as residence sites for acetylcholine:

1. Morphological evidence shows that small agranular vesicles occur in cholinergic nerves to the sphincter muscle of the rabbit iris (Richardson, 1964).
2. Nerve endings of the vas deferens containing only agranular vesicles were not osmiophilic after incubation in Krebs-Hanseleit solution containing a high concentration of norepinephrine (Thoenen et al., 1966).
3. Electronmicrographs of the neuromuscular junction of skeletal muscle (Birks et al., 1960) where the first evidence for acetylcholine as a neuromuscular transmitter accrues (Dale, Feldberg, and Vogt, 1936) reveal the presence of small clear vesicles in the axon terminus.
4. Studies of brain tissue homogenates subjected to gradient

centrifugation to isolate synaptosomes indicates that clear vesicles contain acetylcholine (de Robertis et al., 1963; de Robertis, 1967).

Consequently, proper conditions of fixation which preserve the granular cores can facilitate differentiation of adrenergic from cholinergic fibers. Glutaraldehyde and postomication has been recommended for more reliable fixation of dense core vesicles (Thoenen and Tranzer, 1967) but this method is still often ineffective (Geffen and Livett, 1971). Later a second procedure was developed which relied on glutaraldehyde fixation followed by potassium dichromate which improved preservation of dense-core vesicles when applied to adrenergic nerves leading to the vas deferens (Tranzer et al., 1969). According to an extensive review by Geffen and Livett (1971), glutaraldehyde and osmium tetroxide are unreliable; however, either fixation with osmium tetroxide without prior treatment with aldehyde fixation, or fixation with glutaraldehyde followed by incubation in potassium dichromate and postomication are both procedures which can account for preservation of about 50% to 80% of the dense core vesicles respectively associated with adrenergic axons. The dichromate technique was developed by Barnett and Wood (1964) and the electron dense deposit observed is the result of oxidation of epinephrine or norepinephrine by the dichromate.

The fact that electron lucent secretory vesicles were present in the axon at sites of all close neuromuscular apposition regardless of how the sinus was fixed (glutaraldehyde and OsO_4 ; perfusion with OsO_4 glutaraldehyde, potassium dichromate, and OsO_4) leads one to conclude that even though conditions

were ideal for preservation of granular cores, none of the axons could be considered adrenergic or serotonergic. All the axons observed with electron microscopy probably represent the cholinergic fibers whose presence was demonstrated by the histological thiocholine method in this dissertation.

Although no granular vesicles could be found in axon profiles next to muscle cells, small numbers of the larger granular vesicles (850\AA in diameter) were present in parts of axons which would be unlikely sites for neuromuscular transmission, e.g., in axon bundles heavily invested with connective tissue and other cellular elements of the endoneurium. These large granular vesicles (LGV) have been found in presynaptic terminals of autonomic ganglia (Taxi, 1961) as well as in sympathetic nerve endings on smooth muscle (Elfvin, 1963; Grillo and Palay, 1962; Richardson, 1962, 1964). Their rather common distribution in both cholinergic and adrenergic nerves is therefore a useless criterion for distinguishing between these two classes of nerve. The occurrence of LGV's in the sinus nerves of the Petromyzon is therefore difficult to assess. Although the LGV's may contain a neurotransmitter, their role remains obscure. They are almost twice the size of the smaller granular vesicles (SGV) which are thought to contain norepinephrine and are not labelled after the administration of tritiated norepinephrine (Budd and Salpeter, 1969).

However, Tranzer and Thoenen (1968), using the cytochemical technique of Barrnett and Wood (1964) in an investigation of the iris and vas deferens innervation of cats, concluded that the LGV of the adrenergic nerve endings contain biogenic amines, most probably norepinephrine, whereas the LGV of the cholinergic nerves do not contain these amines.

In the present work, since the site along the axon where the clear vesicles are most numerous and the site at which the distance between axolemma and sarcolemma is reduced to under 500\AA are coincident, it can be assumed that this is the point where neuromuscular transmission occurs. During the course of this investigation, at least one neuromuscular contact could be found that conformed to the strict criteria of neuromuscular contiguity in the heart as defined by Thaemert (1970). This definition specifies that the space between muscle membrane and nerve cell membrane must not be occupied by a basal lamina, a situation which normally occurs in the mouse heart when that space is reduced to within 40mu . The existence of one such contact would suggest the existence of more but they were difficult to demonstrate. However, numerous neuromuscular appositions between $400\text{-}500\text{\AA}$ were demonstrated in which the basal lamina from both nerve and muscle cells was present. The high density of secretory vesicles within the axon at these points would indicate that the area of neuromuscular contiguity was at least nearby. The presence of large families of vesicles at points where the basal laminae lie within a $400\text{-}500\text{\AA}$ space between nerve and muscle tempt the investigator to wonder whether neuromuscular transmission can occur at these points as well as at the contiguities. Since the basement membrane at the skeletal muscle endplate is a continuous structure (Birks, Huxley, and Katz, 1960), it would seem that the basement membrane may not be effective in preventing neuromuscular transmission. Nevertheless the specific absence of basement membrane at a particular site may represent a preferred point of depolarization along the sarcolemma.

Evidence for neurotrophic influences separate from those exerted by the neurotransmitter (Guth, 1968), along with information suggesting that there

may be more than one such substance exerting its effects on the sarcolemma (Albuquerque, 1971), make it necessary to postulate sites of release and action for such hypothetical substances. Such substances may be capable of exerting their effects across the basal lamina. Nevertheless, the complete understanding of the role of the basal lamina in neuromuscular relationships remains to be explained.

The positive chronotropic response to vagal stimulation and/or to the administration of acetylcholine (Augustinsson et al., 1956; Falck et al., 1966; Jensen, 1969; Otorii, 1953) still remains the most interesting aspect of the lamprey heart. Normally such a response to vagal stimulation or acetylcholine can only be elicited from hearts pretreated with atropine (Brown and Maycock, 1942; Hoffmann, 1945; Hoffmann, Hoffmann, Middleton, and Talesnik, 1945) which has long been known to paralyze the "muscarinic" or slowing action produced by acetylcholine (Dale, 1914). Similar sympathomimetic effects produced by acetylcholine in other animals led some researchers to suggest that the cardio-acceleration is the result of stimulation of sympathetic structures within the heart (Burn, Leach, Rand, Thompson, 1959) and was the suggested explanation for this phenomena in the Petromyzon (Augustinsson et al., 1956). On the other hand atropine is without effect on the lamprey heart (Augustinsson et al., 1956; Falck et al., 1966) and the only receptors thought to exist in the Petromyzon myocardium are nicotinic (Falck et al., 1966).

Consequently the explanation for cholinergic acceleration in the Petromyzon could come from either the concept of cholinergic mediated adrenergic responses or from the idea that there exists an acceleratory receptor for acetylcholine.

Such an acceleratory receptor of acetylcholine might be a necessity in lampreys in conjunction with the relative refractory nature of cyclostome hearts to catecholamines (Bloom et al., 1961; Ostlund, 1954), both of which may have been developed as a compensating mechanism for the consistently high concentrations of adrenaline present in cyclostome cardiac tissue. Since a discreet adrenal medulla is not present in cyclostomes, chromaffin tissue is distributed throughout the circulatory system with a large population of chromaffin cells in the heart.

If the idea of cholinergic mediated adrenergic responses is considered, it can be discussed in terms outlined in the Burn and Rand (1959) or Koelle (1961) hypotheses. According to Burn and Rand, acetylcholine may be responsible for the release of norepinephrine from peripheral storage sites such as catecholamine containing cells or nearby adrenergic axons. Koelle suggests a role for acetylcholine at the presynaptic membrane where it causes the release of sufficient additional acetylcholine or norepinephrine to produce the characteristic postsynaptic effect.

These theories have a sound background. In experiments on atropinized heart-lung preparations of the dog and cat, large doses of acetylcholine produce epinephrine like effects, i.e. an increase in frequency and amplitude of the heart beat. Furthermore, the perfusate from these hearts collected during the action of acetylcholine produces a positive inotropic effect on the hypodynamic frog heart (Hoffman, 1945).

Nicotine was shown to produce the same results as acetylcholine in the isolated rabbit atria pretreated with atropine to abolish the "muscarinic" or

inhibitory response of these drugs. In the presence of Hexamethonium, stimulation produced by these drugs is suppressed (Kottegoda, 1953).

Burn and Rand (1958) repeated these experiments on the rabbit with identical results but found that atropinized atria treated with reserpine, which depletes the rabbit heart of catecholamines and inhibits norepinephrine synthesis by inhibiting uptake of its precursor dopamine (Bertler, 1956; Rutledge, 1967) are not stimulated by nicotine.

Similar mechanisms were observed for other tissues (for references see Burn and Rand, 1960, 1965) leading Burn and Rand to conclude that in all organs with a sympathetic innervation, acetylcholine and nicotine acting peripherally produce the same effect as stimulation of the sympathetic nerves and that this effect is dependent upon the liberation of the norepinephrine since the effect is abolished after degeneration of the sympathetic nerves and/or treatment with reserpine (Burn and Rand, 1962).

Since cyclostome hearts contain very high concentrations of catecholamines (Euler and Fange, 1961; Ostlund, 1954), the positive chronotropic response of lampreys to acetylcholine may involve liberation of norepinephrine or epinephrine from the fluorescent catecholamine cells (Falck et al., 1966) and has been suggested by Augustinsson (1956) for Petromyzon whose content of epinephrine is higher than other cyclostomes (Augustinsson et al., 1956; Bloom, 1961; Falck et al., 1966; Mazeaud, 1969; Ostlund, 1960).

At least one site of catecholamine storage which could be under cholinergic control can be eliminated from consideration in Petromyzon marinus since fluorescence microscopy does not reveal an adrenergic contribution to the

heart (Falck et al., 1966). In fact, fish hearts seem to lack adrenergic innervation in general (Randall, 1968).

If cholinergic neurons are responsible for the actual liberation of adrenaline from the catecholamine containing cells, direct innervation of chromaffin cells would be expected. Electron micrographs described in this dissertation failed to show evidence of direct innervation of the dense-core vesicle containing cells even though many such cells were observed in the sinus venosus. Furthermore, based on silver impregnation and the histological localization of ChE, the total distribution of nerve in the Petromyzon marinus heart seems to be confined to the sinus region whereas catecholamine cells are profusely distributed in the ventricle and atrium as well.

It is possible, however, that administered acetylcholine could act on the catecholamine containing cells which would release enough adrenaline to effect the myocardial cells. However, if the cytological relationships are kept in mind, the abundant innervation of myocardial cells established in this text seems to call for a more direct influence of nerve upon in vivo cardiac muscle function in this heart. Some further doubt can be raised concerning the acetylcholine induced liberation of catecholamines in the lamprey since the hagfish, another member of the cyclostome family, which also contains large amounts of catecholamines (Euler, 1961; Ostlund, 1954) is refractory to acetylcholine.

Certainly the biphasic response to cardiac stimulation has been well investigated and apparently involves cholinergic-adrenergic interaction at least in mammals. In the non-atropinized isolated cat atria-nerve preparation,

stimulation of postganglionic cardiac fibers from the stellate ganglion or stimulation of the vagus nerve results in a biphasic chronotropic and inotropic response (Leaders, 1963). Maximal chronotropic and inotropic responses to vagal stimulation occurred first followed by a period of cardiostimulation. Corresponding to this, maximum positive chronotropic and inotropic responses to sympathetic nerve stimulation occurred first followed by a period of cardioinhibition. It was concluded by the author that he had demonstrated the ability of cholinergic nerves to influence adrenergic nerve activity and vice versa since: 1) pretreatment of animals with reserpine abolished both the positive chronotropic response to sympathetic nerve stimulation and the secondary positive chronotropic response to parasympathetic nerve stimulation, and 2) the negative chronotropic response following sympathetic nerve stimulation was not abolished by reserpinization, but was inhibited by administration of HC-3 and vagal nerve stimulation.

This biphasic interaction is most likely operative in and responsible for the mechanism of vagal escape since catecholamine depletion by reserpine (Campos and Friedman, 1962; Roberts and Stadter, 1960) and acute cervical transection combined with bilateral stellate ganglionectomy with or without reserpine or an adrenergic neurone blocking agent delays the tendency of the heart to escape from vagal suppression (Campos and Friedman, 1962).

However, pharmacological aspects of the lamprey heart were reviewed by Falck (1966) in relation to the Burn and Rand theory. He noted that normally nicotine produces a biphasic response in the isolated mammalian heart: an initial negative inotropic and chronotropic effect followed secondly by a

positive inotropic and chronotropic effect. Both responses are blocked by ganglion blocking agents (Kottegoda, 1953). Since the initial response is blocked by atropine, the first phase of the nicotinic effect is due to stimulation of cholinergic receptors. Since the second response is blocked by beta-receptor blocking agents, the second phase represents stimulation of adrenergic receptors. According to Burn and Rand, this secondary phase is due to the release of catecholamines from storage sites of the heart.

However, Falck goes on to note that nicotine does not produce a biphasic response in the isolated lamprey heart. Instead nicotine causes positive chronotropic and negative inotropic effects which are unaffected by hexamethonium. Acetylcholine produces similar effects to that of nicotine which are not antagonized by atropine but the effects due to both acetylcholine and nicotine are blocked by curare. Thus the only cholinergic receptors in the lamprey heart appear to be curare sensitive since muscarine and atropine are ineffective pharmacologically in this preparation. Since Falck could show that even larger doses of acetylcholine could not elicit sympathomimetic effects after blocking the only existing cholinergic receptor with curare, he concluded that catecholamines in the lamprey heart are not released by nicotine or acetylcholine.

The second phase due to acetylcholine administration, namely the negative influence on the force of the beat is particularly interesting if one is to suggest that such an effect is due to release of catecholamines, especially so since catecholamines are known to elevate adenylcyclase (Drummond, 1966; Hammermeister, 1965; Murad, 1962; Namm, 1968; Robison, 1965), the enzyme

catalyzine synthesis of adenosine-3', 5'-monophosphate (C-AMP) which is believed to be responsible for the positive inotropic effects due to catecholamines (Sutherland, 1960, 1968) and as the possible adrenergic receptor (Robinson, 1967). One would expect positive inotropism if acetylcholine or vagal stimulation released catecholamines in this heart.

If the presence of abundant catecholamine containing cells within the heart results in a normally elevated concentration of adrenaline, there may be no direct nervous control required as far as their release is concerned. This consistently high adrenaline concentration in the myocardial tissue may override the inhibitory effects of vagal stimulation thus resulting in acceleration. However, the direct innervation of the myocardial cells themselves suggests a direct role for these nerves in causing the accelerated effect. The only known accelerating effect that can be attributed to acetylcholine in the heart is its ability to shorten the time required for repolarization (Burgess and Terroux, 1953). This characteristic acceleration of repolarization has been demonstrated for vagal stimulation also (Hoffman and Suckling, 1953).

The well known acceleration of cardiac pacemaker depolarization by adrenaline (Hauswirth, 1968) could be assisted by vagal stimulation. Since the increased rate of pacemaker depolarization is dependent upon the resting membrane potential such that when it is reduced there is a corresponding reduction in the rate of rise of the action potential, acetylcholine would play an important role in returning the muscle membrane to a re-established condition of threshold where elevated adrenaline may again initiate an accelerated depolarization.

The possibility that acetylcholine is working on a "nicotinic" or accelerating receptor seems unjustified since high frequencies of stimulation (60 impulses/sec) of the median jugular vein which carries the nerves that innervate the heart, results in a biphasic response. The first part of the response is the usual accelerating effect but this is followed by a second component, a period of bradycardia. This secondary retarding phase, revealed only at higher frequencies of stimulation, could be accounted for by a higher concentration of acetylcholine released at the muscle cell which would supercede the effect of catecholamines and exert the cardioinhibitory effects normally observed in other vertebrate hearts.

Certain cytological relationships would seem to be required for the expression of a biphasic response to vagal stimulation. If the initial response is cardioinhibitory, it would seem necessary that the myocardial cells be directly innervated by cholinergic fibers. If this initial response is followed by a period of cardiac acceleration, it would be logical to assume that vagally released acetylcholine acts on a peripheral adrenergic site, based on material of Burn and Rand (1959), sufficiently close such that the transmitter can effect that site and yet far enough away from the myocardial cell such that cholinergically released catecholamines exert their effects secondary to or after the direct effect of acetylcholine on the muscle cell. It is generally believed that this interaction would be most feasible between the cholinergic and adrenergic neurons since the cholinergic and adrenergic innervations of most mammalian hearts have large areas of congruent distribution (Ehinger, Falck, Persson, and Sporrang, 1968), and since the vagus is known to contain at

least some adrenergic fibers (Muroybashii et al., 1968; Nielson, Owman, and Sabantini, 1969). The other possible sites are the special catecholamine cells found in the cardiac ganglia of some mammals (Jacobowitz, 1967).

On the other hand if the response to vagal stimulation at low frequencies is monophasic as described by Falck (1966) and Augustinsson et al. (1956) for the lamprey but positively chronotropic, two cytological explanations seem to be most possible: 1) vagal innervation of peripheral catecholamine containing cells whose release of epinephrine or norepinephrine is directly responsible for cardioacceleration, or 2) direct vagal innervation of muscle cells in an environment of high catecholamine concentration due to the presence of a diffuse adrenomedullary system within the heart. The first explanation was suggested for Petromyzon (Augustinsson et al., 1956) but the results of the present investigation can only support the second point since no innervation of chromaffin cells was found amidst a highly innervated musculature. The direct innervation of the muscle without an intervening nerve-catecholamine cell relationship also suggests a contributing role rather than a passive one for the nerves in causing acceleration.

B. MYOFILAMENT CHOLINESTERASE ACTIVITY

The positive reaction of the A-band to incubation in acetylthiocholine medium was not unexpected since it had been demonstrated previously in rat cardiac muscle (Karnovsky, 1964) and since purified myosin extracts had been shown to have cholinesterase activity, hydrolyzing thiocholine esters (Kover and Kovacs, 1957) used as substrates in biochemical reactions for cholinesterase.

The myosincholinesterase activity is associated with the lighter or L-fraction of meromyosin (Kover, Kovacs, and Konig, 1957; Kiss, Szigeti, and Varga, 1954) which exists throughout the A-band except for the M-band and is most prominent nearer the A-I junction as indicated by antigen-antibody fluorescence (Finck, Holtzer, and Marshal, 1959). This distribution is similar to that shown in Figure 33 of this text.

Biochemical data indicates that myosincholinesterase from rat cardiac muscle is a non-specific cholinesterase, hydrolyzing butyrylthiocholine more readily than acetylthiocholine (Kover and Kovacs, 1957; Ord and Thompson, 1950, 1951; Girardier, Baumann, and Posternak, 1960). These results for rat cardiac muscle were duplicated cytochemically with the A-band showing higher reactivity with butyrylthiocholine than with acetylthiocholine (Karnovsky, 1964).

The A-band from the Petromyzon marinus heart, however, exhibits overlapping substrate preferences with slightly more reactivity toward acetylthiocholine, a result which also is supported by biochemical assays on cardiac homogenates from Petromyzon (Augustinsson et al., 1956).

An interesting discrepancy observed between the myosincholinesterase activity displayed by the rat and that observed for the lamprey is their sensitivity to N-ethylmaleimide, a sulfhydryl group blocker. N-ethylmaleimide (NEM) did not effect the A-band reaction of rat cardiac muscle (Karnovsky, 1964) but completely eliminated the A-band reaction of the Petromyzon heart as described in this text. Karnovsky suggested that NEM be used to control the reduction of ferricyanide by free sulfhydryl groups that might be present in tissue sections (Mirsky and Anson, 1936a, 1936b) which would result in a false reaction. NEM as a sulfhydryl group blocker is a logical control since it

leaves unaffected the activity of the cholinesterases which are not sulfhydryl-dependent enzymes (Boyer, Laidy, and Myrback, 1959; Hargreaves, 1955; Mounter and Whittaker, 1953). However, since sulfhydryl groups are important for maintaining the structural stability of many molecules (for refs., cf. Cecil, 1963) it is possible that alkylation of the free sulfhydryl groups by NEM resulted in a conformational change thereby altering structural requirements for cholinesterase activity along the myosin filament. NEM could also have bound to sulfhydryl groups that may have been adjacent to the active center responsible for the cholinesterase activity and remained attached to present an obstacle sterically hindering access of the substrate to that site. H-meromyosin possesses a number of sulfhydryl groups, about half of which are free to react with NEM (Szent-Gyorgyi, 1947). If the active center for the ChE activity on the L-meromyosin molecule is sufficiently close to the point of attachment of H-meromyosin, the latter explanation might easily be true.

Whether or not myosincholinesterase actually functions within the cell to hydrolyze acetylcholine is still a lesser problem than trying to understand what effect internally produced acetylcholine could exert on the filaments. Although it is generally agreed that the attachment of H-meromyosin to L-meromyosin is a flexible one and that therefore some complex macromolecular reorganization occurs between either these units of myosin or between H-meromyosin and actin during the contractile process, it is not specifically clear what they are (Huxley, 1969). Until this information is available, interpretation of the cholinesterase activity associated with L-meromyosin may be impossible. The only speculation that this student can bring to bear is that intracellularly produced acetylcholine may modulate muscle activity by affecting the

filaments some way. Such activity could be studied by isolation of intact filaments according to the method of Hanson and Huxley (1951) and studying their ability to contract under the polarized light microscope with varying conditions of acetylcholine and anticholinesterase.

C. SIGNIFICANCE OF SUBSARCOLEMMA CISTERNAE IN PETROMYZON MYOCARDIAL CELLS

Since cholinesterase activity found associated with the lamprey myocardial cell is confined to the sarcoplasmic reticulum, the role of the sarcoplasmic reticulum in cardiac muscle is reviewed briefly as an introduction to the section discussing myocardial cell cholinesterase.

Following Huxley's classical experiments in which elicitation of I-band contractions occurred with microelectrode placement on the cell membrane near the Z-band and his subsequent correlation of I-band recruitment along an axis occupied by a transverse element of the sarcoplasmic reticulum (Huxley, 1958), it is now generally accepted that these T-tubules act as a conduction pathway for the intracellular spread of electrical activity from the fiber surface to the interior of the cell (Franzini-Armstrong, 1964; Huxley, 1964; Peachy, 1965; Ruska and Edwards, 1958; Smith, 1966). This theory depends heavily on the ultrastructural demonstration of T-tubule continuity with the sarcolemma, first demonstrated in cardiac muscle (Forssmann and Girardier, 1966; Leak, 1970; Linder, 1957; Nelson and Benson, 1963; Raynes et al., 1967, 1968; Simpson, 1965; Simpson and Oertelis, 1961, 1962).

Terminal cisternae or saccular elements of the longitudinal sarcoplasmic reticulum are found closely applied to these T-tubules in cardiac muscle (Fawcett, 1961; Fawcett and McNutt, 1969; Simpson and Oertelis, 1962; Simpson and

Rayns, 1968; Sommer and Johnson, 1970). This morphological relationship represents the site of excitation-contraction coupling.

It is known that activation of the contractile mechanism of isolated myofibrils and actomyosin systems require calcium ions (Niedergerke, 1959; Podolsky, 1961; Weber, 1963) which are sequestered and stored in the terminal cisternae of the sarcoplasmic reticulum of intact muscle (Cohen, 1969; Constantin, 1965; Legato, 1969; Pease, 1965). Associated with the depolarization of the sarcolemma in skeletal and cardiac muscle there is a release of calcium ions from the terminal cisternae and diffusion to the filaments initiating contraction (Langer, 1965; Niedergerke, 1963; Winegrad, 1968). Retrieval of calcium by the sarcoplasmic reticulum is associated with relaxation of the muscle (Ebashi, 1961, 1962; Hasselbach, 1964).

The common denominator displayed by all the longitudinal elements of the sarcoplasmic reticulum is their indirect contact with the muscle cell membrane which is most often made through the transverse tubular invaginations of the sarcolemma. Evidence suggests, however, that the necessity for T-tubules is governed by the width of the muscle fiber and the distance between the innermost myofibrils and the sarcolemma. In other words, the ionic gradients known to occur during depolarization of the normal cardiac muscle fiber (Page, 1962) apparently are not effective in causing contraction in large muscle fibers with disrupted T-tubules. In such fibers an action potential is recorded without an accompanying contraction (Gage and Eisenberg, 1967). Even before the existence of transverse invaginations of the sarcolemma was known, it has been calculated that the limited rate of diffusion of calcium required for the

contractile process could not account for the rate and synchrony of myofibril contraction in large muscle cells (50-150 μ in diameter) (Hill, 1948). However, the demonstration of a lack of T-tubules in frog ventricular myocardial cells which are extremely small in diameter (less than 5 μ across) (Staley and Benson, 1968) suggests that T-tubules may not be necessary if the myocardial cells are sufficiently small in diameter and the longitudinal segments of the sarcoplasmic cells are sufficiently close to the sarcolemma forming diads where excitation-contraction coupling can occur.

Further ideas supporting this line of reasoning come from a study of early postnatal psoas muscle in the rat. Immediately after birth when the muscle diameter is small, the T-tubules penetrate only shallowly into the muscle cell, triads are peripherally located, and frequent diadic associations of the sarcoplasmic reticulum with the sarcolemma are observed. One to two weeks later a marked proliferation of the sarcotubular system accompanied by deeper penetration of T-tubules is associated with an increase in fiber diameter and the number of myofibrils (Schiaffino and Margeth, 1969). Although most vertebrate cardiac myofibers show the presence of both internal couplings (S.R. in close association to T-tubules) and peripheral couplings (S.R. associated with sarcolemma) (Fawcett, 1969; Sommer and Johnson, 1968), those without T-tubules always have the peripheral coupling (Johnson and Sommer, 1967; Jewett, 1971).

This logic can be applied to the myocardial cells of the Petromyzon marinus. This investigation indicates the lack of a T-tubule system as in other species of cyclostome (Bloom, 1962; Leak, 1969) but the presence of numerous

diadic associations of the sarcoplasmic reticulum (subsarcolemmal cisternae) occurring subjacent to the sarcolemma at the I-band. From the above treatment it is suggested that both the small fiber width of Petromyzon myocardial cells (5-10 μ) and the peripheral location of the myofilaments near the sarcolemma apparently make T-tubules unnecessary for excitation-contraction coupling. It is suggested that the peripheral coupling is sufficient for excitation-contraction coupling in Petromyzon marinus since the farthest distance measured between sarcolemma and the innermost myofilament is only about 1 micron.

It is a qualitative estimation that the subsarcolemmal cisternae are more extensively developed in myocardial cells of the sinus venosus than in the atrium and ventricle of the lamprey. The obvious implication is that functions normally attributable to these sarcoplasmic reticulum segments occur to a greater degree in myocardial cells of the sinus than in the atrium or ventricle. For instance, if calcium binding is evenly distributed within sarcoplasmic reticular membranes, then it might be reasonable to suggest that the subsarcolemmal cisternae of the sinus would be capable of releasing greater amounts of calcium ions during depolarization of the sarcolemma than less developed segments of sarcoplasmic reticulum in the atrium and ventricle. Since the rate of contraction of isolated myofibrils has been shown to increase with calcium ion concentration (Podolsky, 1961), it may be possible that sinus myocardial cells require a higher concentration of calcium ions during contraction than atrial and ventricular muscle. Although electrophysiological properties are the primary reason for the shorter time required for depolarization of pacemaker tissue as in the sinus venosus than for the atrium (Hutter and Traut-

wein, 1956), there may be an elevated Ca^{++} ion requirement to facilitate the mechanical part of the contractile mechanism too. Although no quantitative data is available on the relative amounts of sarcoplasmic reticulum in atrial muscle versus sinus muscle cells, sufficient data is available to compare the amounts of sarcoplasmic reticulum utilized in excitation-contraction coupling of ventricular myocardial cells with that found in twitch skeletal muscle which develops peak tension more rapidly. Stereological measurements of the components involved in excitation-contraction coupling have demonstrated quantitatively that both the total sarcotubular membrane area per unit cell volume and the total contribution of terminal cisternae to the total sarcotubular volume in frog twitch muscle (Peachy, 1965) exceed corresponding values for rat ventricular muscle (Page et al., 1971).

D. POSSIBLE ROLE OF MYOCARDIAL CELL ACETYLCHOLINESTERASE IN CARDIAC AUTOMATICITY

Previously cholinesterase activity had been localized cytochemically in the cardiac sarcoplasmic reticulum of the rat (Karnovsky, 1964b) and the embryonic rabbit (Hagopian et al., 1969). Ultrastructural deposition of reaction product in the rabbit heart was demonstrated in tissue treated with tetraiso-propylpyrophosphoramidate but absent from tissue treated with B.W.284C.51. In addition, butyrylthiocholine substrate substitution for acetylthiocholine gave no reaction. From these substrate and inhibitor specificities, the authors concluded that acetylcholinesterase was the specific enzyme present in the sarcoplasmic reticulum of rabbit myoblast.

On the other hand Karnovsky (1964b) found that the intensity of the reaction was greatest with butyrylthiocholine than with acetylthiocholine and that reactivity to both substrates could be eliminated with eserine (10^{-4} M) and DFP (10^{-5}). It was concluded that the reaction in the rat heart was due to a non-specific cholinesterase which was in accord with biochemical observations on the rat heart (Girardier et al., 1960; Kover and Kovacs, 1957; Ord and Thompson, 1950, 1951). The substrate and inhibitor specificities demonstrated in Petromyzon marinus paralleled those exhibited by the embryonic rabbit suggesting that the S.R. enzyme in the lamprey is also AChE, a conclusion seconded by biochemical evidence (Augustinsson et al., 1956) for the lamprey. The elimination of the reaction by eserine excludes the possibility of aliesterase action on the substrate. Furthermore, B.W.284C.51, which is highly specific for acetylcholinesterase, blocked the reaction totally whereas pretreatment with tetraisopropylpyrophosphoramidate failed to inhibit deposition of reaction product. The fact that enzymatic activity was insensitive to tetraisopropylpyrophosphoramidate is substantiated by the relative failure of incubation in butyrylthiocholine to produce a reaction product which would have been indicative of non-specific cholinesterase.

The cytological site of acetylcholinesterase activity in the lamprey and rabbit myocardial cell S.R. is somewhat confusing because previous demonstrations of this enzyme in muscle were always associated with nerve as in the motor endplates of skeletal muscle (Koelle, 1963). Because nerve degeneration of skeletal muscle results in a general disappearance of acetylcholinesterase from the endplate, which returns with reinnervation, the presence of the enzyme

at this site is apparently under neurotrophic control (for refs. cf. Guth, 1968). However, the presence of the enzyme within the lamprey ventricular myocardial cells which are not directly innervated at the stage investigated in this dissertation, suggests a purely myogenic controlled function of the enzyme. Although it is still unknown what function might be performed by acetylcholinesterase in the S.R. of cardiac muscle, the presence of this enzyme in the embryonic rabbit heart prior to innervation led Hagopian et al., (1969) to suggest that an ACh/AChE system of myogenic origin may be responsible for regulation of the heart beat exclusive of nervous influence, an idea conceived by Bulbring and Burn (1949). Although this explanation is rather vague, it has been the only one offered thus far.

Bulbring's theory was based on the following experimental results. Freshly excised auricles of the rabbit heart beating in Tyrode's solution respond to the addition of acetylcholine by a depression in rate. The auricle will remain beating normally for 24-36 hours and then become hypodynamic. Acetylcholine will restart such quiescent auricles, returning the beat to its normal rate and amplitude. However, further addition of acetylcholine will exert its usual negative inotropic and chronotropic effects. Further, dried powder of freshly excised rabbit auricles incubated according to Feldberg and Mann (1946) synthesized about two and a half times more acetylcholine than powder prepared from quiescent auricles. The addition of acetylcholine to the former diminished synthesis of acetylcholine while it augmented synthesis in the latter. The ability to acetylate choline via choline acetylase fell to a low in auricles which stopped beating whereas choline acetylase activity rose to

the initial value displayed by freshly excised auricles restarted with acetylcholine. This led the authors to conclude that the rhythmic activity of the auricular muscle and synthesis of acetylcholine within it are inseparably linked.

It has been shown that inhibition of choline acetyl transferase (choline acetylase) by acetylcholine occurs in vitro at a concentration of 10mM increasing to 100mM. The inhibition was found to be competitive for choline and non-competitive for acetyl-CoA (Kaita and Goldberg, 1969).

It would seem that such a negative feedback mechanism of acetylcholine on choline acetylase would be heavily dependent on AChE, the enzyme responsible for hydrolysis of acetylcholine. For instance, it is known that AChE is inhibited by excess acetylcholine (Augustinsson and Nachmansohn, 1949). If excessive amounts of acetylcholine are present to overload AChE, then it might be expected that the unhydrolyzed ACh would depress choline acetylase activity (ChAc), whereas efficient hydrolysis of ACh would produce enough choline to elevate or maintain the level of ACh synthesis. As such AChE would regulate the activity of ChAc de facto.

If such an intricate internal mechanism is operating within myocardial cells and depends upon acetylcholine and the enzymes for its synthesis and hydrolysis, it is difficult to suggest how such a mechanism works, what it accomplishes, and how it fits in with ionic gradients known to be associated with depolarizing cardiac muscle cells (Pate, 1962) or external methods of control such as vagal stimulation (de Castillo and Katz, 1955) and acetylcholine application (de Castillo and Katz, 1954; Hoffmann and Suckling, 1953).

Since no ideas have been proposed for the explanation of an ACh/AChE mechanism within the myocardial cell, speculation of a possible interaction of AChE and ATPase may be in order based on such an in vitro observation in nerve tissue. The basis for applying some of the results of these observations on nerve to cardiac muscle rests on two points: 1) a close association has been demonstrated between glycolytically produced ATP and the transmembrane potential of guinea pig papillary muscle (McDonald et al., 1971), and 2) the enzyme responsible for hydrolysis of ATP has been detected in the sarcoplasmic reticulum of cardiac muscle in the dog (Sommer and Spach, 1964) and rat (Essner and Quintana, 1963) and more recently ATPase has been localized ultrastructurally to the lateral elements of the S.R. lying against T-tubules and to the subsarcolemmal cisternae of rat myocardial cells (Rostgaard and Behnke, 1965). This ultrastructural localization common to both AChE and ATPase is important since a strong interrelationship between AChE and ATPase has been established at least for rat brain microsomes in vitro (Kometiani and Kalandarishvili, 1969). These authors have shown that acetylcholine reduces the activity of Na-K-ATPase significantly. The inhibition of Na-K-ATPase by acetylcholine is reversed by physostigmine in concentrations sufficient to completely inhibit microsomal AChE without influencing ATPase activity itself. This suggests that ACh does not act on ATPase but rather through some effect on AChE. Conversely ATP and ADP, both substrates for ATPase, lowers the activity of AChE also, but only in the presence of Na^+ , K^+ , and Mg^{++} ions suggesting that ATP does not influence AChE directly but through some effect of Na-K-ATPase. This mutual inhibition of substrates of AChE and AChE led the authors to conclude that AChE is some-

how involved in the mechanism of active transport of cations and specifically suggest that acetylcholine therefore acts to inhibit ATPase driven active transport of sodium and potassium ions. Although the exact involvement of AChE in such an interrelationship with ATPase is unknown, it is interesting to speculate that AChE could limit the amount of acetylcholine available within the myocardial cell for inhibition of ATPase. Certainly it is a demonstration that AChE could be at least indirectly involved in maintenance of membrane potential. Alternately, since the S.R. is responsible for retrieval of calcium during muscle relaxation and this calcium uptake is stoichiometrically related to ATP hydrolysis (Hasselbach, 1965), perhaps acetylcholine may exert inhibition of this particular ATPase and therefore modulate the energy driven accumulation of calcium ions by the S.R.

It would appear that an ACh/AChE mechanism within myocardial cells performs functions of myogenic control as opposed to external influences directed on the myocardial cell by cholinergic nerves. In attempting to distinguish between these myogenic and neurogenic functions, anticholinesterases could be used. And in fact the effects of a number of anticholinesterases have been studied on the isolated rabbit auricle (Burn and Kottegoda, 1953) and on the Starling heart lung preparation in the dog (Burn and Walker, 1954). In both cases eserine (10^{-8} M) usually decreased the rate of contractions or arrested them completely. Auricles arrested by eserine became inexcitable by electrical stimulation but the beat resumed again if eserine was removed from the bath. In higher concentrations (10^{-8} - 10^{-4} M), eserine augments the amplitude of contraction and lower concentrations (10^{-3} M) decrease the amplitude (Burn and Kotte-

goda, 1953). Burn and Walker reasoned that since anticholinesterases retard the destruction of acetylcholine, that the slowing of the heart rate was due to an increased concentration of acetylcholine formed in the heart and that this acetylcholine was controlling the pacemaker.

In the canine heart-lung preparation diisopropylfluorophosphate (3×10^{-6}) in a concentration which inhibits both pseudo- and acetylcholinesterase (Koelle, 1954) slowed the heart to 67% of its initial value but B.W.284C.51, a specific acetylcholinesterase inhibitor, had no effect. However, it is not known what type of cholinesterase is present in canine myocardial cells whereas in the rabbit auricle where the myocardial cells are known to contain specific AChE (Hagopian et al., 1969), B.W.284C.51 acts like eserine in slowing the heart (Brisco and Burp, 1954). However, lack of information concerning the effect of anticholinesterases on the muscle cell membrane complicate interpretation of these experiments. Since postganglionic parasympathetic nerves are contained even in these isolated preparations, it is impossible to rule out a neurogenic influence on myocardial cell activity as a result of the action of anticholinesterases.

VI. SUMMARY AND CONCLUSIONS

The ultrastructural organization of the young adult lamprey (Petromyzon marinus) has been defined. Light microscopic silver impregnation and cholinesterase localization were used to chart the histological distribution of nerve and myocardial cell cholinesterase within complete serial sections of the Petromyzon marinus heart and the results used to facilitate an electron microscopic search for nervous elements in the heart. Furthermore, Karnovsky's technique for cholinesterase was used to demonstrate the localization of acetylcholinesterase within the myocardial cells themselves. The results were discussed in terms of current theories concerning neurogenic and myogenic control of the heart beat. The results of this investigation are as follows:

1. Although the sinus venosus is tubular and the atrium trabecular in organization, the ventricle is composed of a channel network whose lumens are continuous with the ventricular cavity. Numerous trabeculae arise from the walls of these channels and attach to the epicardium. The three main cell types contained within the endothelial lining of these trabeculae and channels are fibroblasts, dense-core vesicle containing cells or chromaffin cells, and myocardial cells. The chromaffin cells and myocardial cells are surrounded by basement membranes while the fibroblasts are not. The chromaffin cells are always located under the endothelial lining.
2. Silver impregnation indicated an apparent lack of nervous elements in two chambers of the young adult heart, atrium and ventricle. However, the

presence of fine filamentous argyrophilic structures within the sinus venosus indicated that this chamber is well innervated. Consecutive serial sections indicated that these nervous elements were confined to the sinus venosus and did not extend into the atrium or ventricle at this stage of development.

3. The application of Karnovsky's thiocholine method for the histological detection of cholinesterase together with various anticholinesterases indicated that the atrium and ventricle lacked cholinergic innervation but that the sinus venosus was innervated. The sinus contained brown filamentous structures after acetylthiocholine incubation, indicative of acetylcholinesterase activity. The deposition of brown copper ferrocyanide, the reaction product resulting from hydrolysis of the substrate acetylthiocholine by cholinesterase, could be prevented if the tissue was preincubated in 1:5-bis-(4-trimethyl-ammoniumphenyl) pentan-3-one dibromide, a competitive reversible inhibitor of acetylcholinesterase and by eserine sulfate, a group specific reversible inhibitor of cholinesterase. No reaction was observed in sections incubated in the Karnovsky medium lacking substrate.
4. Ultrastructural examination of all three chambers of the heart revealed the presence of nerve in only the sinus venosus. Non-myelinated axons bound in multiples or simply singly within a Schwann cell sheath were distributed within the walls of the sinus. Close associations between nerve axon and myocardial sarcolemma were observed, most often within 400-500Å of each other. At points of neuromuscular association, the axon is found protruding from an incomplete Schwann cell investment with only the basement membranes

of the myocardial cell and the axon-Schwann cell complex separating the two. At these points of neuromuscular association, profuse electron lucent vesicular profiles morphologically characteristic of cholinergic secretory vesicles were found crowded against the inside of the axon membrane closest to the myocardial cell. No nervous contacts were observed with catecholamine containing cells although axons completely ensheathed in Schwann cells could be seen passing in their vicinity. The effect of direct innervation of myocardial cells rather than of catecholamine containing cells is discussed with regard to the positive chronotropic response of the lamprey heart to vagal stimulation.

5. Application of Karnovsky's technique for the ultrastructural localization of cholinesterase indicated the presence of the electron dense reaction product, copper ferrocyanide, at two sites: 1) the thick myofilaments, especially near the A-I junction, and 2) the sarcoplasmic reticulum. The reaction within the A-band was attributed to the myosin-cholinesterase activity of L-meromyosin and could be blocked by preincubating the tissue in N-ethylmaleimide, a sulfhydryl group blocker. The reaction of the A-band was overlapping but slightly greater with acetylthiocholine than with butyrylthiocholine.

The subsarcolemmal cisternae were highly reactive after incubation in acetylthiocholine but virtually no reaction was detected after butyrylthiocholine incubation. After acetylthiocholine incubation a dense particulate deposit could be found on the membranes of the subsarcolemmal cisternae. These focal points were often separated from one another along the membrane of about

150A. Electron dense deposits were also found within the lumens of these subsarcolemmal cisternae. In either case the reactivity seemed to be confined to a section of subsarcolemmal membrane along the I-band with other parts of the sarcoplasmic reticulum much removed from this point being unreactive. Unlike the reaction of the A-band filaments to acetylthiocholine incubation, the reactivity of the subsarcolemmal cisternae was not prevented by N-ethylmaleimide. Formation of the reaction product could not be prevented by tetraisopropylpyrophosphoramidate, an irreversible inhibitor of non-specific or butyrylcholinesterase, but was eliminated by preliminary incubation in B.W.284C.51, a specific reversible inhibitor of acetylcholinesterase, and by eserine sulfate, a group specific cholinesterase. The role of intramyocardial acetylcholinesterase is discussed with regard to myogenic control of muscle contraction.

It is concluded that the Petromyzon marinus heart is cholinergically innervated and that cholinergic fibers first enter the heart within the first three months after termination of the ammocete state, i.e. at the earliest stage of development of the young adult. Furthermore, these nerve fibers enter the heart through the cartilagenous pericardium where the median jugular and ventral hepatic veins join the sinus venosus; no nerve fibers were observed to enter the heart via the duct of Cuvier. These nerve fibers are distributed to the sinus venosus but do not extend beyond this chamber into the atrium or ventricle at this stage of development. Nerve axons were observed to make numerous neuromuscular associations but no innervation of the chromaffin cells which comprise the diffuse adrenomedullary system was detected.

The localization of myocardial cell cholinesterase in the Petromyzon

marinus in the sarcoplasmic reticulum closely associated to the sarcolemma is further evidence that myocardial cholinesterase may be involved in modulation of sarcolemma function.

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PLATE I

Diagram:

Lateral view of *Petromyzon Marinus* Heart. V, ventricle; A, atrium; S, sinus venosus; mj, median jugular vein; hv, hepatic vein; dC, duct of Cuvier; p, cartilagenous pericardium; sa, sino-atrial orifice; av, atrio-ventricular orifice; va, ventral aorta.

Figure 1:

Cross-section of *Petromyzon marinus* ventricle fixed with formalin and stained with haematoxylin and eosin. A large endocardial channel (C) can be seen directed toward the epicardium (E) and bifurcating into two channels of smaller dimension. Sandwiched between the two smaller channels are muscle trabeculae (T) cut in cross-section. Some horizontally-cut trabeculae can be observed to take origin from channel walls (solid arrow) and others to attach to the epicardium (empty arrow). 280X.

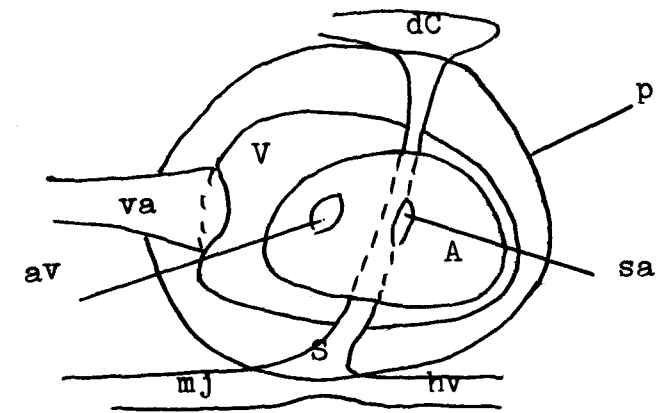
DIAGRAM: Lateral view of *Petromyzon marinus* heart

PLATE II

Figure 2:

Endocardium of ventricular cavity as viewed with scanning electron microscope. The endothelial cells (E) lining the ventricular cavity are continuous with the endothelial lining of the endocardial channel whose lumen (L) opens into the ventricular cavity. This particular channel is approximately 15u in diameter. Such channels are responsible for the intraventricular circulation since coronary arteries are lacking. 3850X.



Figure 2

PLATE III

Figure 3:

The ventricular endothelial lining is typically represented here with the endothelial cell nucleus (N) constituting the widest portion and the cytoplasmic extensions (E) the thinnest. The endothelial extensions are approximately .1 μ thick and represent the only barrier between the intertrabecular spaces (S) and the myocardial cells (M). 14,000X. uranyl acetate and lead citrate.

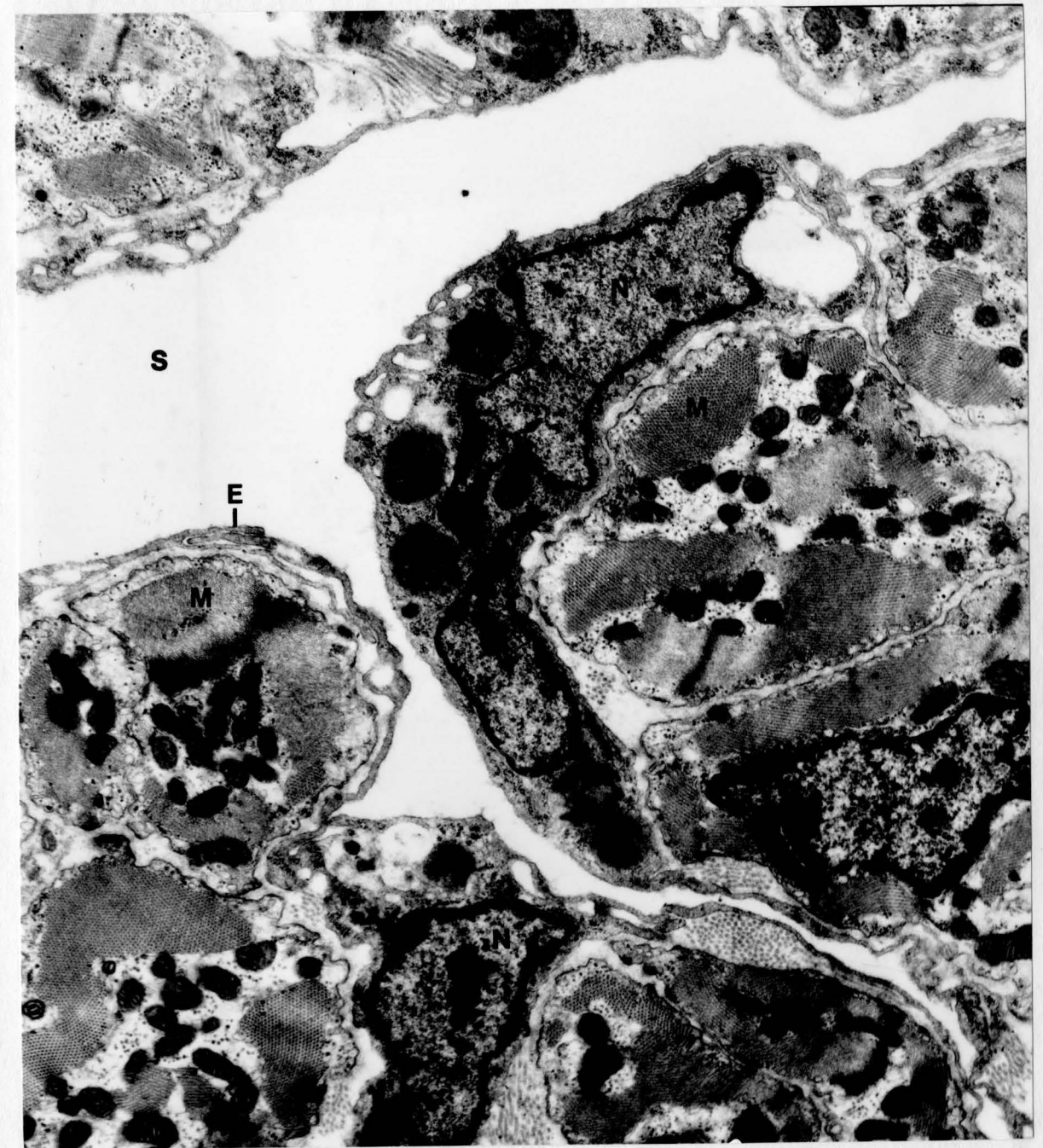


Figure 3

PLATE IV

Figure 4:

The three major cell types occurring in the myocardium, myocardial cell (M), chromaffin cell (C), and fibroblast (F), are all shown in this electron micrograph of the ventricular myocardium. The chromaffin cell is always deployed immediately next to the endothelial lining (E). The well-developed rough endoplasmic reticulum (arrow) in the fibroblast is a common occurrence. Collagen bundles (X) are usually situated near the endothelial lining as shown here in cross-section. 14,000X. uranyl acetate and lead citrate.

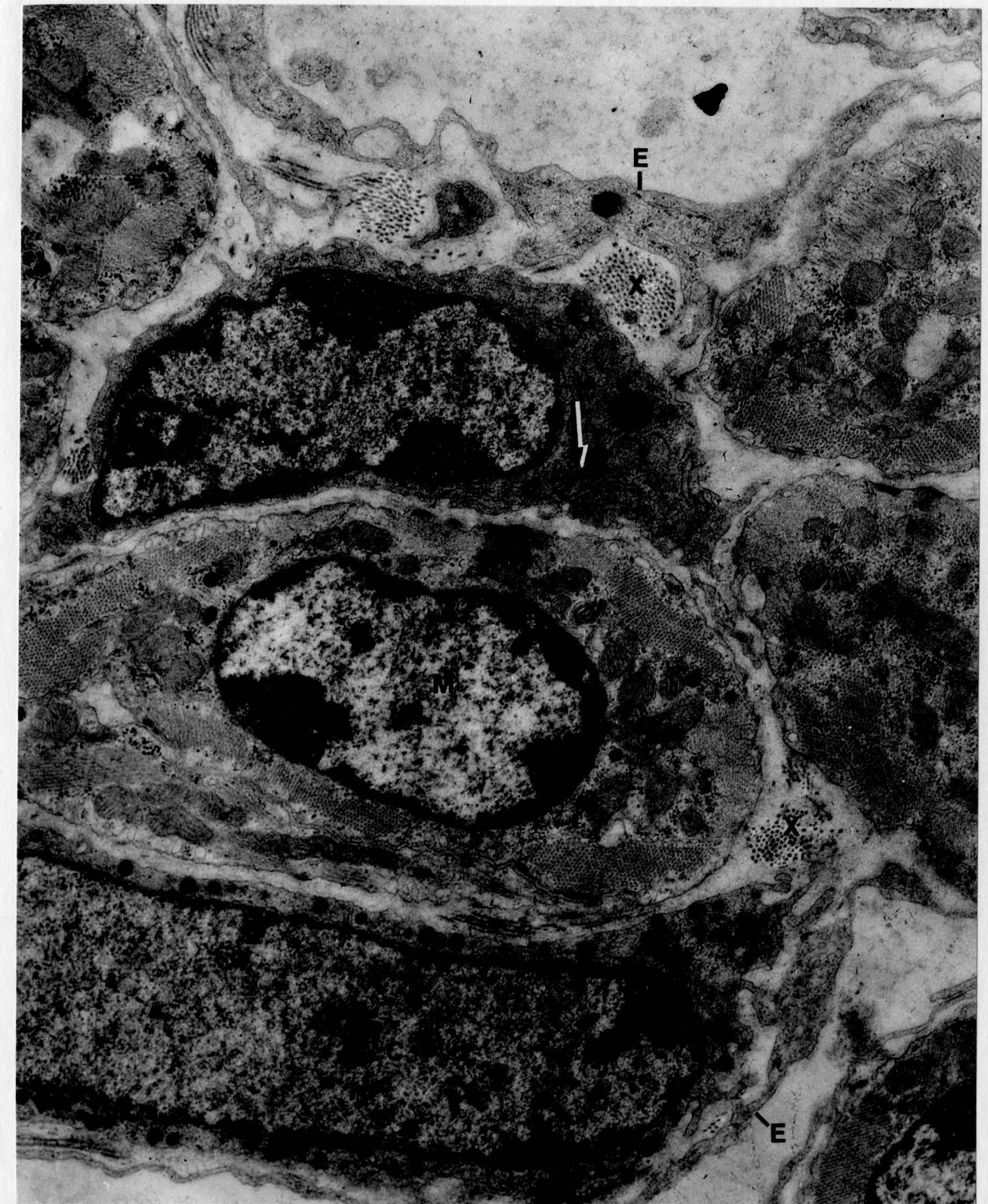


Figure 4

PLATE V

Figure 5:

Ventricular myocardial cells surrounded by a faint basement membrane (empty arrow). Situated immediately under the sarcolemma at the level of the I-band (i) is a segment of sarcoplasmic reticulum (arrow). Z, z-line; A, A-band. 22,000X. uranyl acetate and lead citrate.



Figure 5

PLATE VI

Figure 6:

This atrial chromaffin cell (C) is surrounded by a basement membrane (solid arrow) and deployed immediately adjacent to the endothelial lining (E). Within the chromaffin cell are numerous vesicles (V) containing osmophilic granules of varying electron densities. These vesicles are approximately 1200A in diameter. Dispersed among these vesicles are segments of rough endoplasmic reticulum (empty arrow) and mitochondria. Evidence is highly suggestive that these particular cells synthesize catecholamines. 30,000X. uranyl acetate and lead citrate.

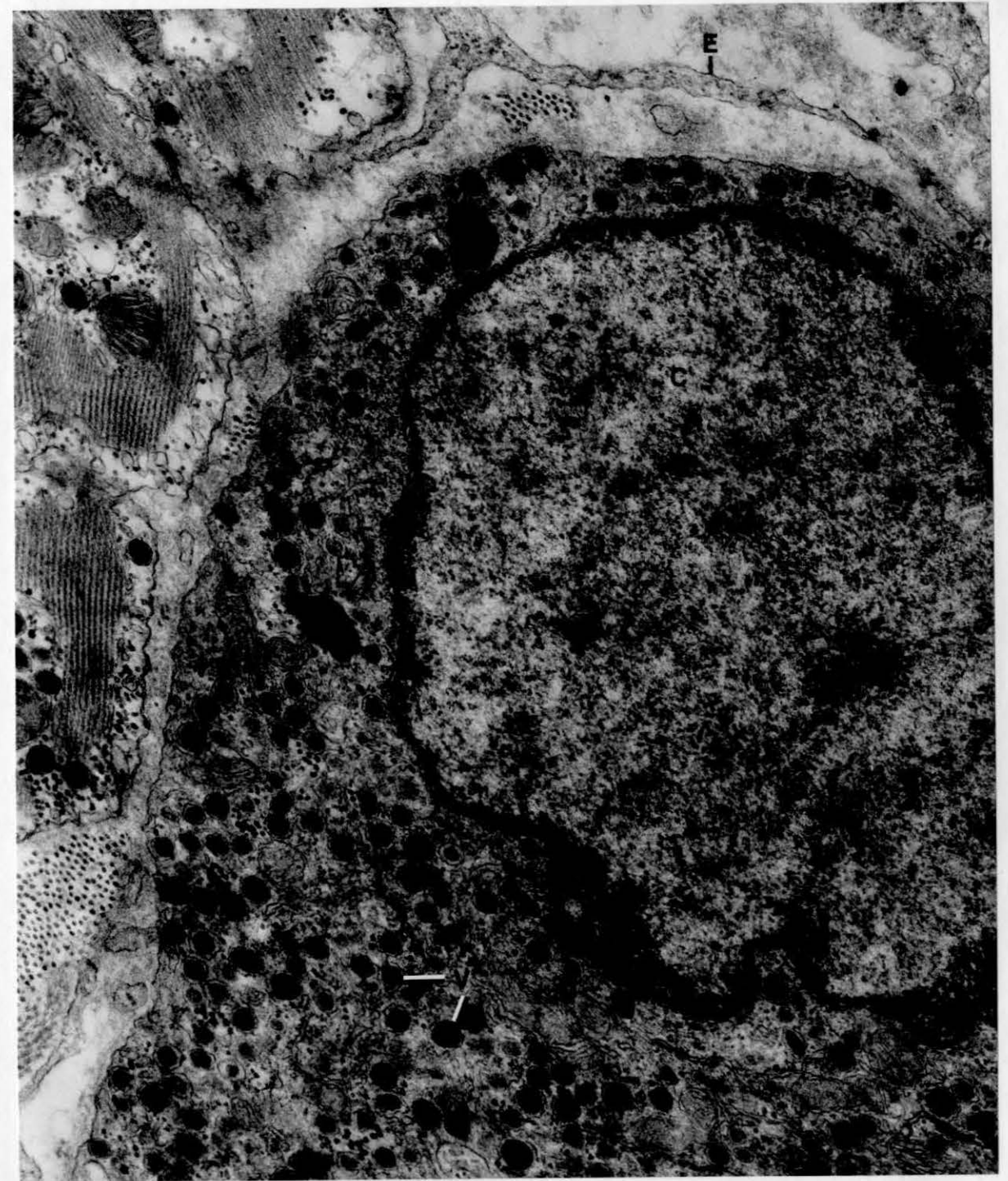


Figure 6

PLATE VII

Figure 7:

The epithelial cells (E) covering the epicardium of the sinus venosus are supported by a well discernible basement membrane (BM). These cells are interlocked at their lateral edges by a corrugated specialization of the cell membrane (empty arrow). A dense aggregation of collagen (C) lies characteristically below the epithelial cells of the sinus. 30,000X. uranyl acetate and lead citrate.

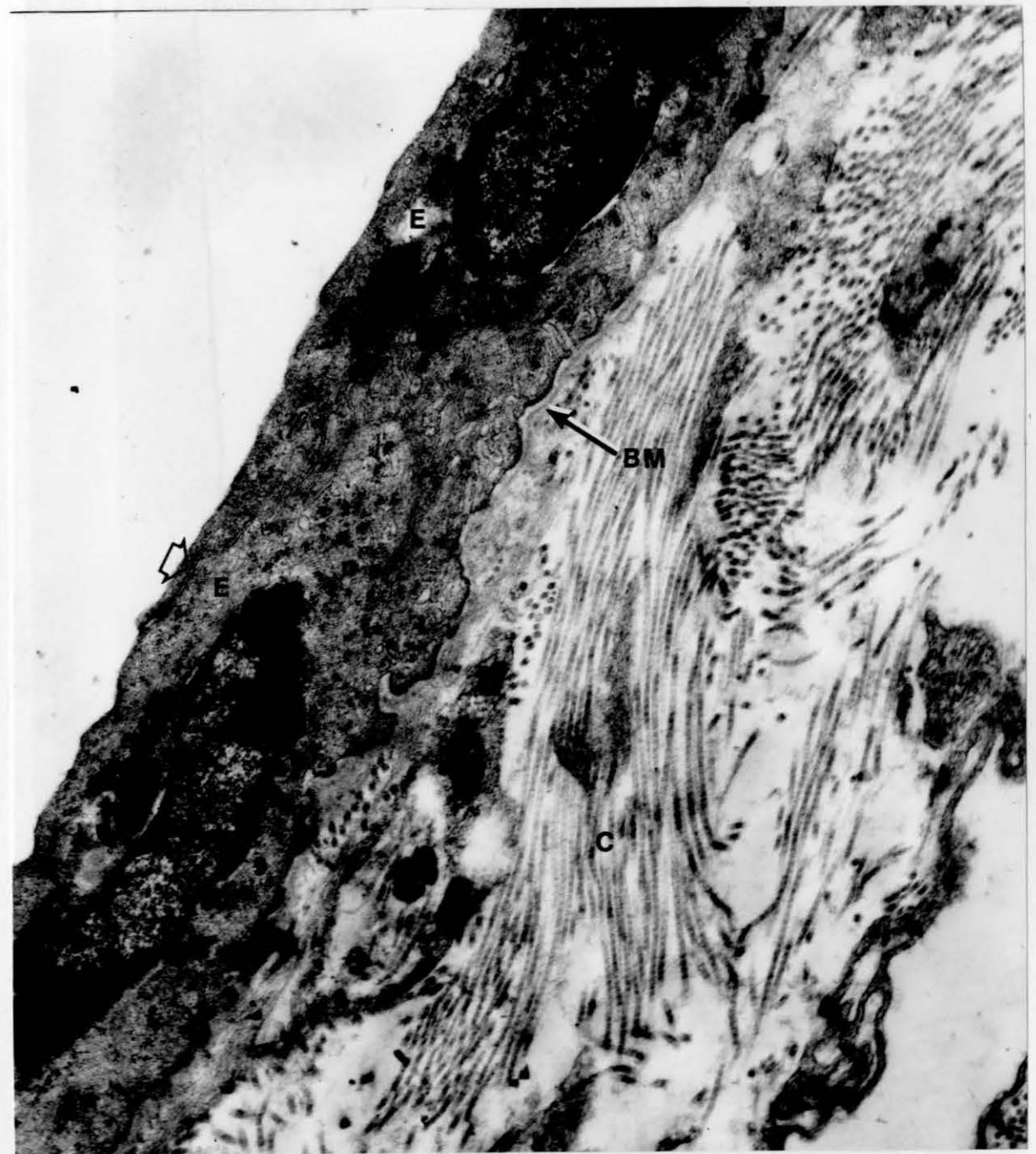


Figure 7

PLATE VIII

Figure 8 & 9:

Adjacent sections of the sinus venosus entering the atrium. Bodian silver impregnation. Argyrophilic nerve fibers (solid arrow) can be observed in the wall of the sinus coursing toward the atrium (A). Note that some fibers (empty arrow) are cut tangentially in the sinus wall (S) but that none pass beyond the sino-atrial valves (sa) into the atrium. There are no nerves stemming from that part of the sinus which drains the duct of Cuvier (dC). The horn of the sinus which contains the nerves in the right of the photographic field drains the median jugular and hepatic veins. Numerous argyrophilic chromaffin cells (C) are distributed throughout all these chambers of the heart and often show a central unimpregnated nucleus. 85X.

Figure 8

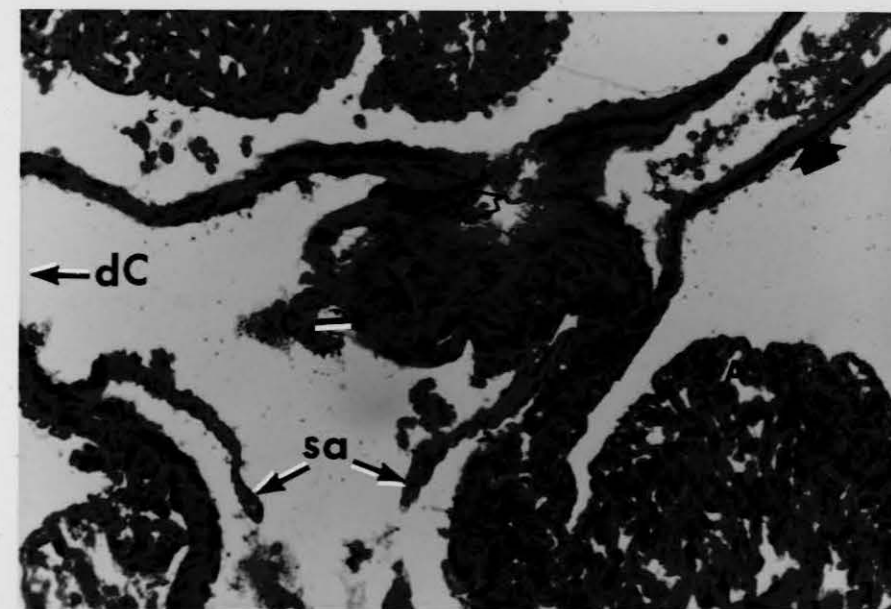
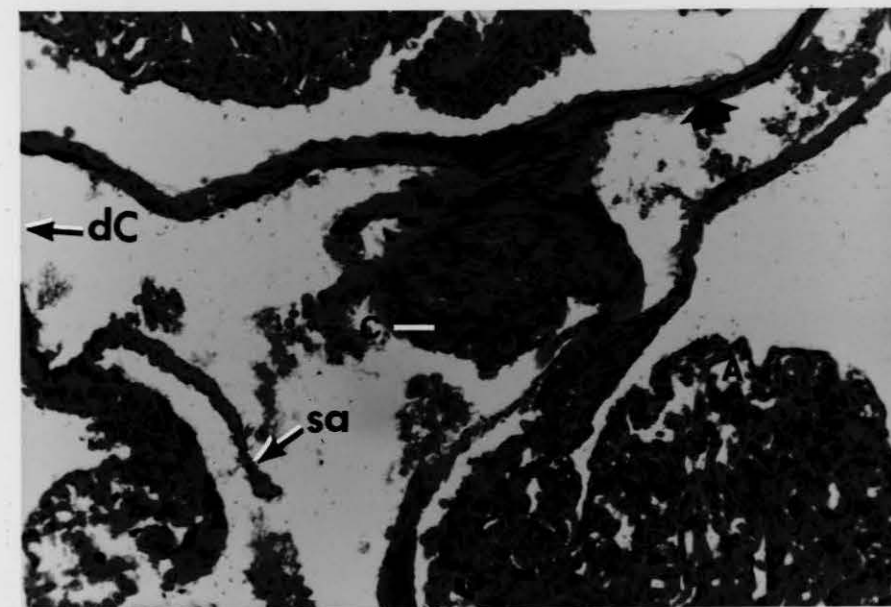


Figure 9

PLATE IX

Figure 10:

Sino-atrial junction. Bodian silver impregnation. No discrete nerve fibers can be distinguished within the wall of the sinus (S) or atrial musculature (A). However, argyrophilic chromaffin cells are present here appearing either spherical with unimpregnated nuclei (empty arrow) or stellate and elongated (solid arrow). The myocardial cell nuclei are also argyrophilic and can be distinguished from the larger chromaffin cells and nerve fibers by their oval shape and smaller size. These muscle cell nuclei are the numerous argyrophilic structures seen best in the ventricle (V) of this figure but are also present in the wall of the sinus. b, blood cells within atrial cavity. 85X.

Figure 11:

Cross-section through the spinal cord at the level of the anterior root. Incubated in Karnovsky-Roots acetylthiocholine medium without anticholinesterase. Note that deposition of copper ferrocyanide, the brown reaction product, has occurred in the ventral motor root (solid arrow), the axons of the spinal cord (SC), and the nerves (empty arrow) innervating the trunk musculature. N, notochord. 85X.

Figure 10

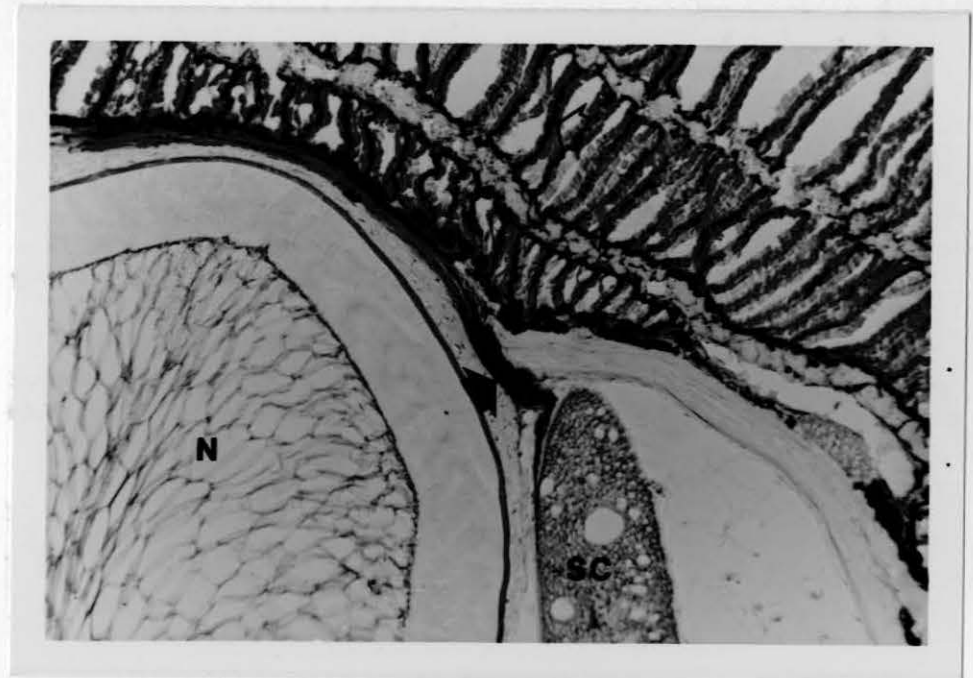
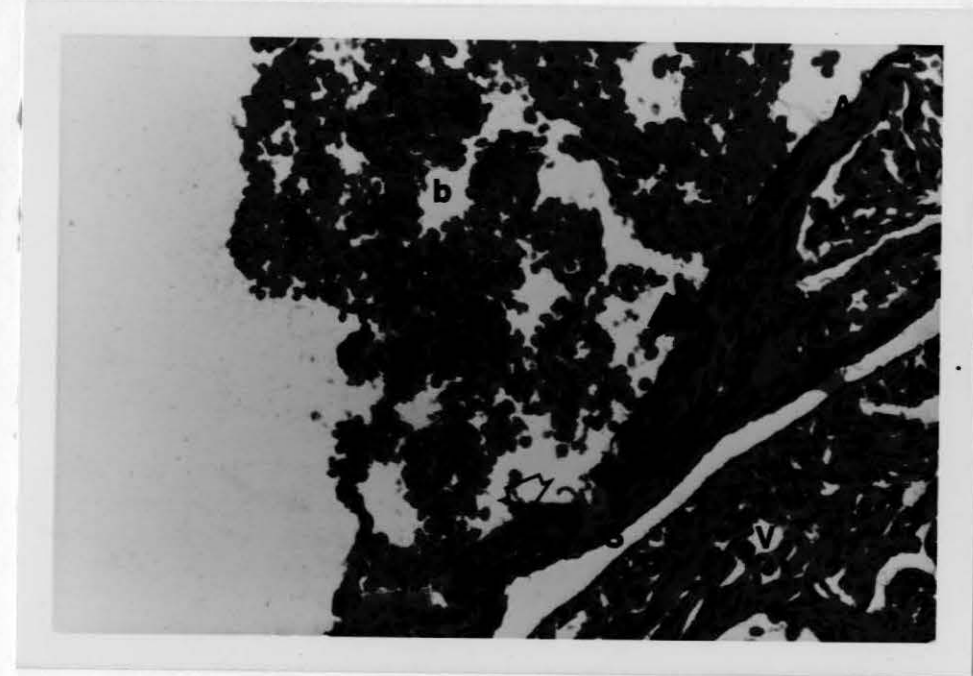


Figure 11

PLATE X

Figure 12:

Cross-section through the spinal cord at the level of the anterior root. Incubated in Karnovsky-Roots medium lacking substrate. No deposition of brown enzymatic reaction product has occurred at the ventral root (solid arrow), in the spinal cord (SC), or the trunk musculature (M). A number of pigment cells (P) are arranged at the base of the trunk musculature. N, notochord. 85X.

Figure 13:

Cross-section through the spinal cord at the level of the anterior root. Incubated in Karnovsky-Roots acetylthiocholine medium with B.W.284C.51, a specific competitive inhibitor of acetylcholinesterase. Reaction has been prevented at the spinal cord (SC), the anterior root (solid arrow), and in the trunk musculature (M). N, notochord. 85X.

Figure 12

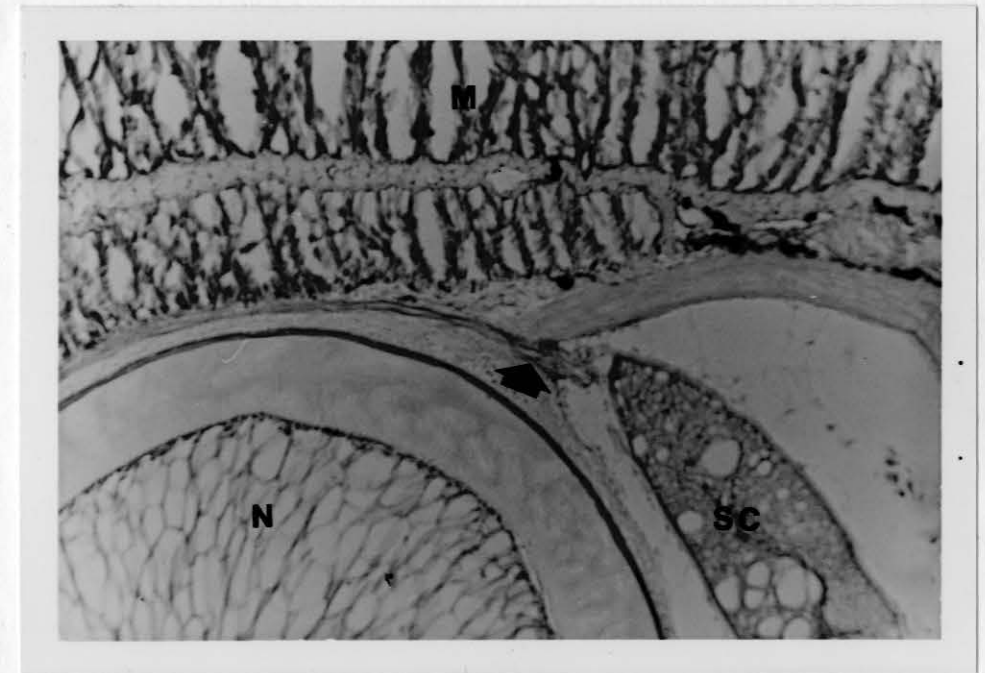
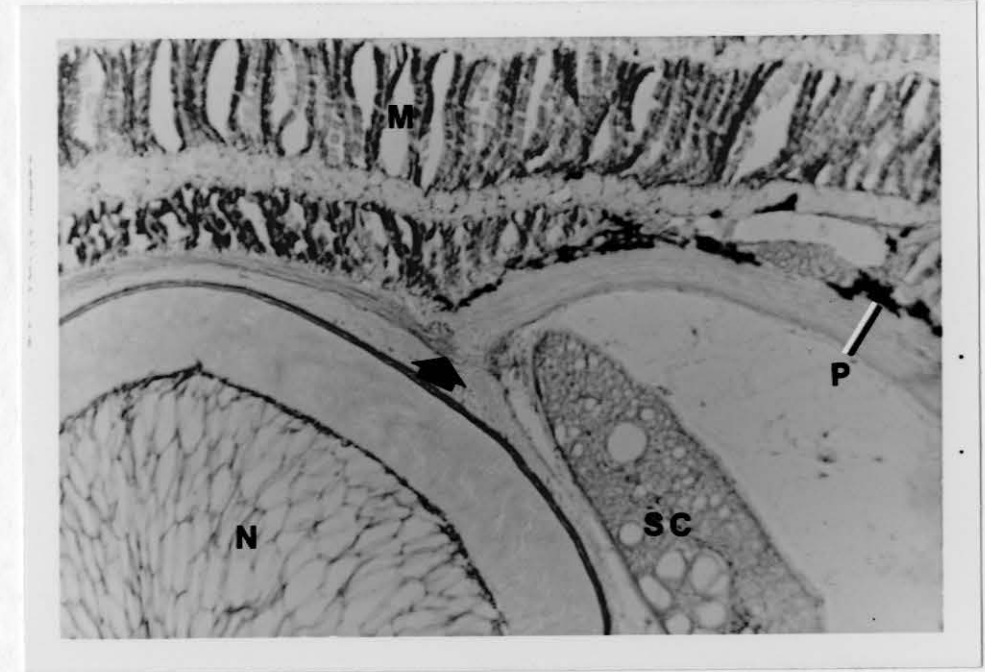


Figure 13

PLATE XI

Figure 14:

Cross-section through the spinal cord at the level of the anterior root. Incubated in Karnovsky-Roots acetylthiocholine medium and eserine sulfate, a group specific inhibitor of cholinesterase. No reaction product is present in the spinal cord (SC) or the ventral root (solid arrow) and approximately 90% of the reaction of the peripheral trunk nerves has been prevented in the trunk musculature (M). 85X.

Figure 15:

Cross-section through the lamprey heart at the level of the sinus venosus. Incubated in Karnovsky-Roots acetylthiocholine medium without anticholinesterases. This slightly enlarged view shows deposition of the copper ferrocyanide on fine nerve fibers in the gill musculature (G) and nerve fibers in the trunk musculature (T). The sinus venosus (arrow) is positively reactive whereas the atrium (A) and ventricle (V) failed to react. 38X.

Figure 14

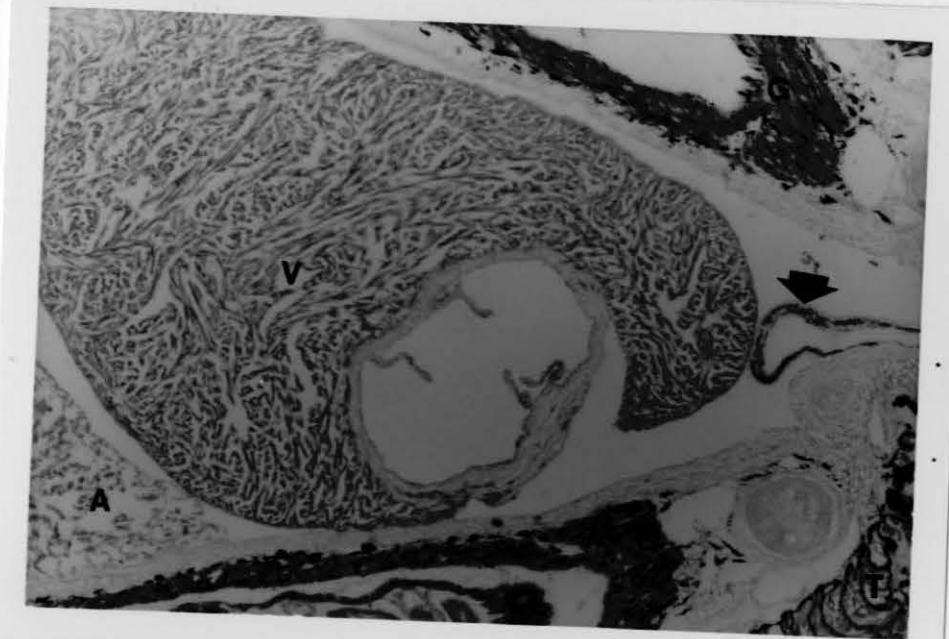
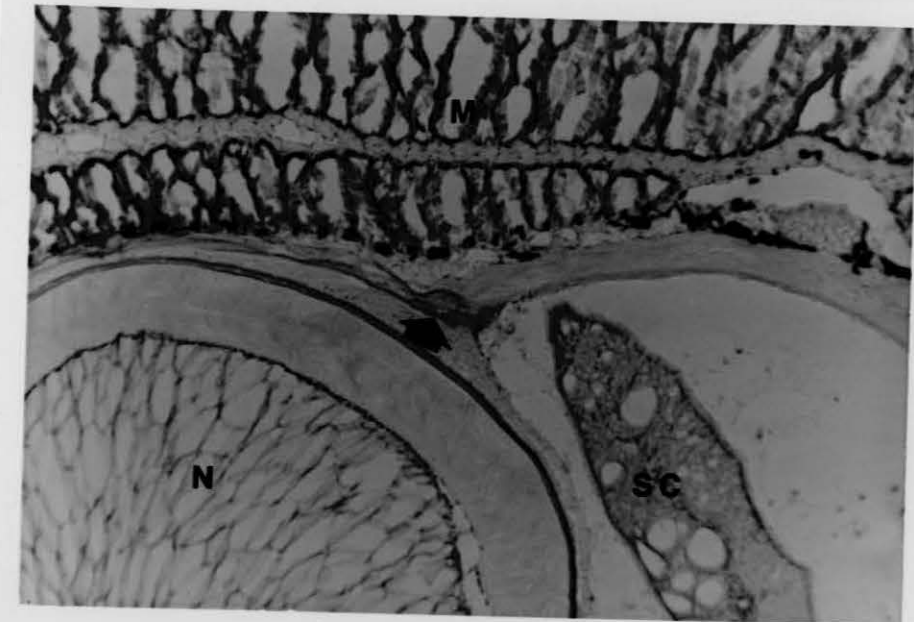


Figure 15

PLATE XII

Figure 16:

Cross-section through the sinus venosus. Incubated in Karnovsky-Roots acetylthiocholine medium without anti-cholinesterases. This higher magnification of Figure 16 illustrates the fine network of positively reacting fibers (arrow) within the sinus (S). Most of the brown reactive fibers are cut in cross-section although some short longitudinal segments are present also. The ventricle (V) is unreactive. P, cartilagenous pericardium. 85X.

Figure 17:

Cross-section through the sinus venosus. Incubated in Karnovsky-Roots medium without substrate. This section is immediately adjacent to the section in Figure 16 but none of the nerve fibers have reacted to produce the brown reaction product. V, ventricle; P, cartilagenous pericardium; S, sinus. 85X.

Figure 16

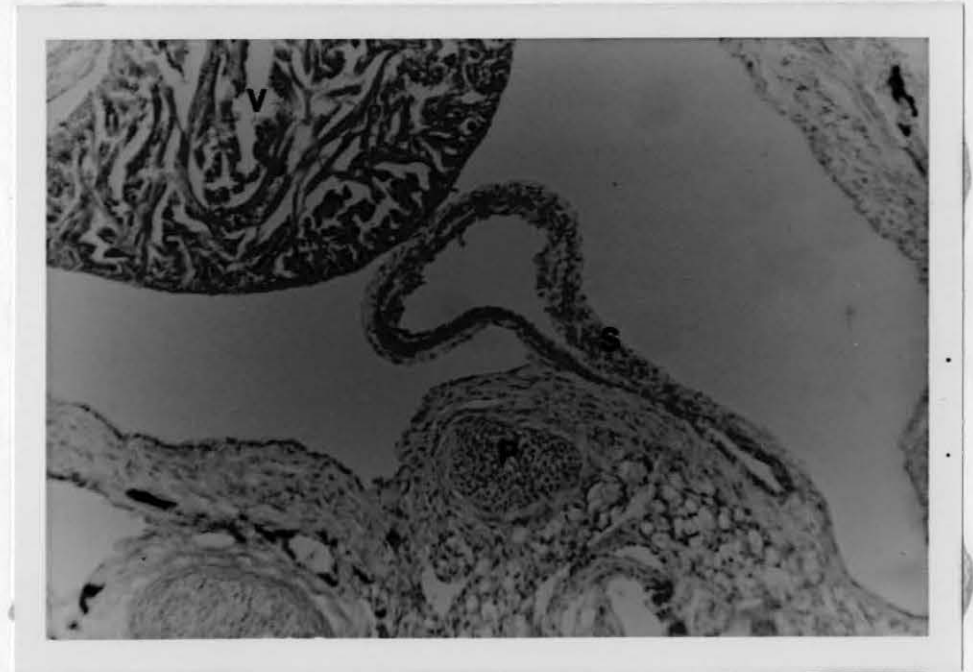
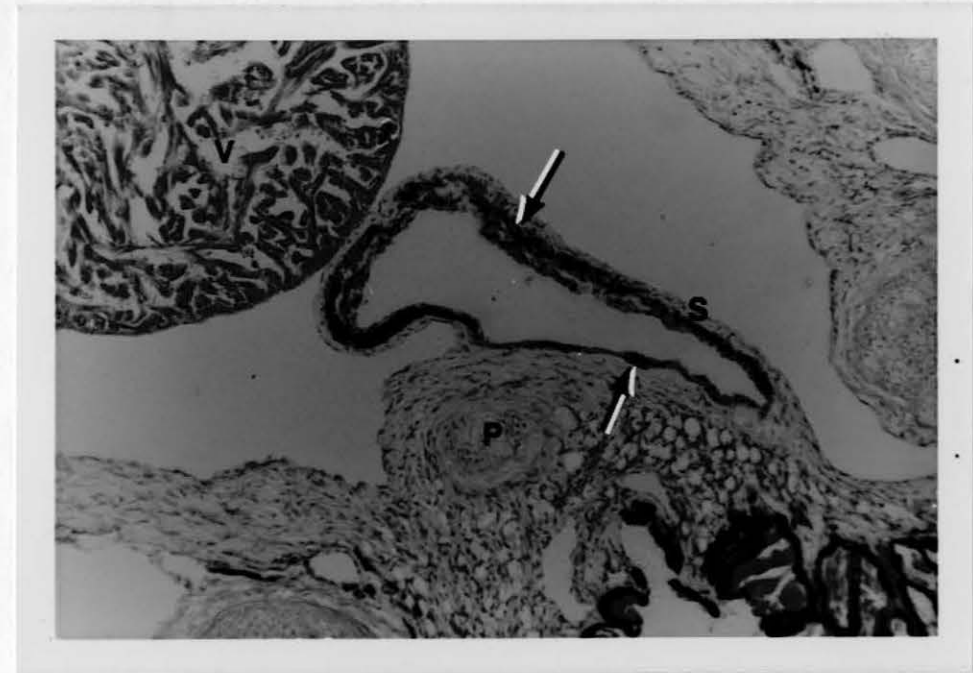


Figure 17

PLATE XIII

Figure 18:

Cross-section through the sinus venosus. Incubated in Karnovsky-Roots acetylthiocholine medium with eserine sulfate, a reversible group-specific inhibitor of cholinesterase. Virtually all reactivity has been prevented in this section through the sinus (S). V, ventricle; P, cartilagenous pericardium. 85X.

Figure 19:

Cross-section through the sinus venosus. Incubated in Karnovsky-Roots acetylthiocholine medium with B.W.284C.51, a specific competitive inhibitor of acetylcholinesterase. No reactive fibers are visible within the sinus (S). V, ventricle; P, cartilagenous pericardium. 85X.

Figure 18



Figure 19

PLATE XIV

Figure 20:

Longitudinal-section through the sinus venosus. Incubated in Karnovsky-Roots acetylthiocholine medium without cholinesterase inhibitors. Brown positively reactive nerve fibers can be observed in longitudinal section (arrow). 38X.



Figure 20

PLATE XV

Figure 21:

Cross-section of nerve bundle within the sinus venosus at point near the junction of the sinus with the median jugular and hepatic veins. A Schwann cell with its nucleus (S) is coiled around a group of axons (a), some of which contain large vesicles about 1000A in diameter with dense cores (arrows). A single larger axon (A) contained within a Schwann cell investment (S) is also present. Cross-sections through the sinus this distant from the atrium commonly revealed the presence of Schwann cell invested axons associated with components of the endoneurium; such as, fibroblasts (F) and dense collagen (C). 48,000X. glutaraldehyde and OsO_4 ; uranyl acetate.



Figure 21

PLATE XVI

Figure 22:

Sinus venosus: A large bundle of axons and a single large axon (A) both surrounded by their respective Schwann cells (S) are further separated from one another by basement membranes (empty arrows) and a thin layer of collagen (vertical arrow). A few large dense core vesicles (solid arrow) are present within some of the axons and resemble similarly appearing vesicles within the chromaffin cell (C) at the bottom of the micrograph. Cellular components of the endoneurium are present (E). 48,000X. glutaraldehyde and OsO_4 ; uranyl acetate.

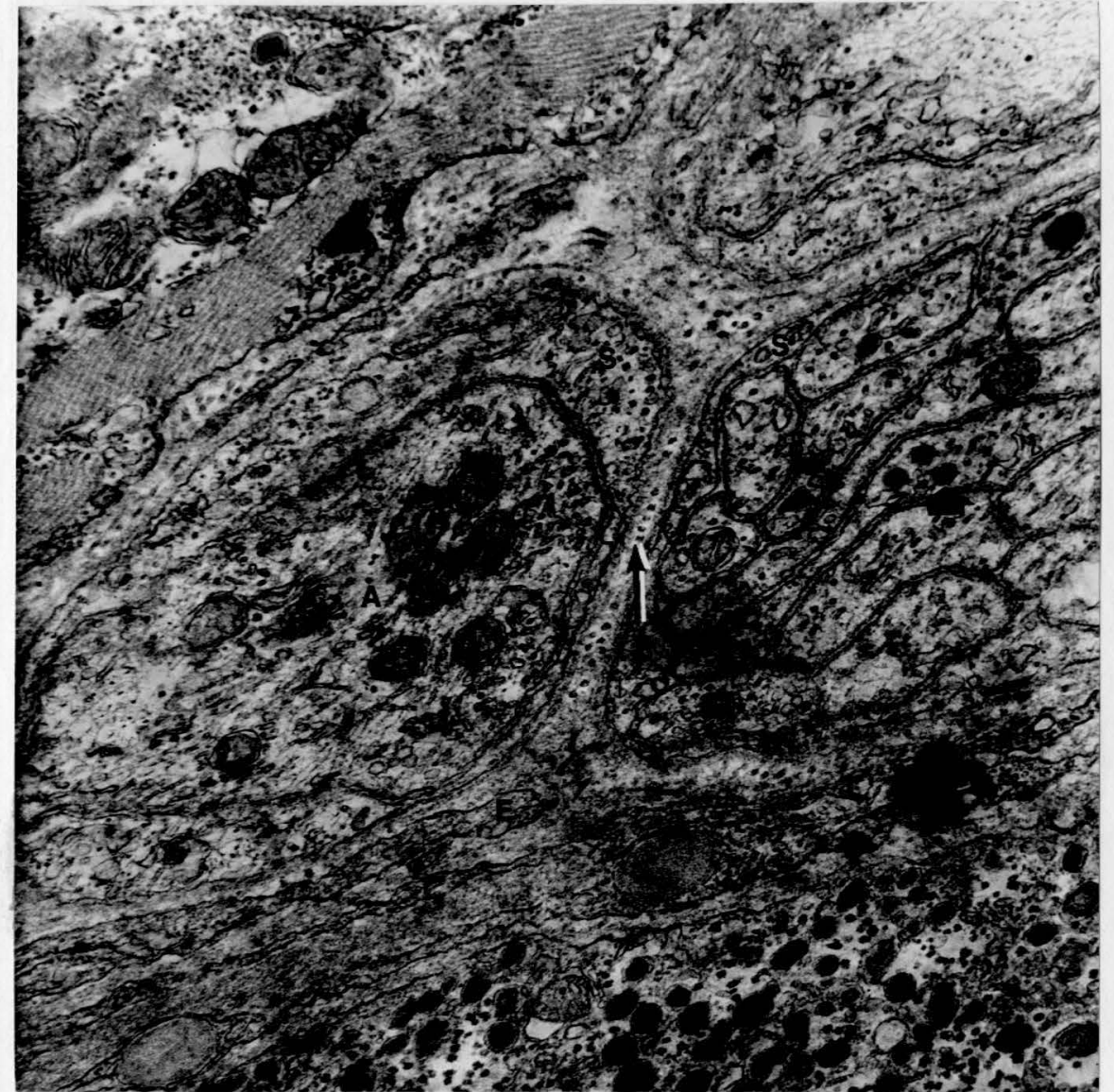


Figure 22

PLATE XVII

Figure 23:

Sinus venosus: A Schwann cell (S) is shown revolved about $1\frac{1}{2}$ times around a single large axon (A) cut in cross-section. A group of axons above and to the left of it are sectioned lengthwise for a short distance. Within one of these longitudinally cut axons are a number of microtubules (empty arrow) cut lengthwise. These microtubules measure about 200A in diameter. Present in addition to the microtubules is a less linear, more densely staining membrane system (solid arrow). 48,000X. glutaraldehyde and OsO_4 ; uranyl acetate.



Figure 23

PLATE XVIII

Figure 24:

Three axons totally devoid of endoneurium shown in the deeper sinus myocardium. Axon "a" is cut in cross-section and surrounded by a single revolution of Schwann cell cytoplasm (S). Within it are two sets of profiles. The less dense profiles (empty arrow) are cross-sections of microtubules and the other more densely staining tubules are cross-sections of membranes similar to endoplasmic reticulum shown in Figure 23. Axon "b" is an axon of similar dimension to axon "a" but cut longitudinally. Axon "c" is only partially circumvented by its Schwann sheath (S). A portion of its axon membrane lies exposed (solid arrow) and is oriented towards an adjacent muscle cell (M). The axon membrane and the sarcolemma are separated by a space of about 450A which is occupied by their basement membranes. This close neuromuscular association is characterized in the axon by the presence of a large family of small electron lucent vesicles (V). m, mesaxon; b, multilaminated body. 30,000X. OsO₄; lead citrate.

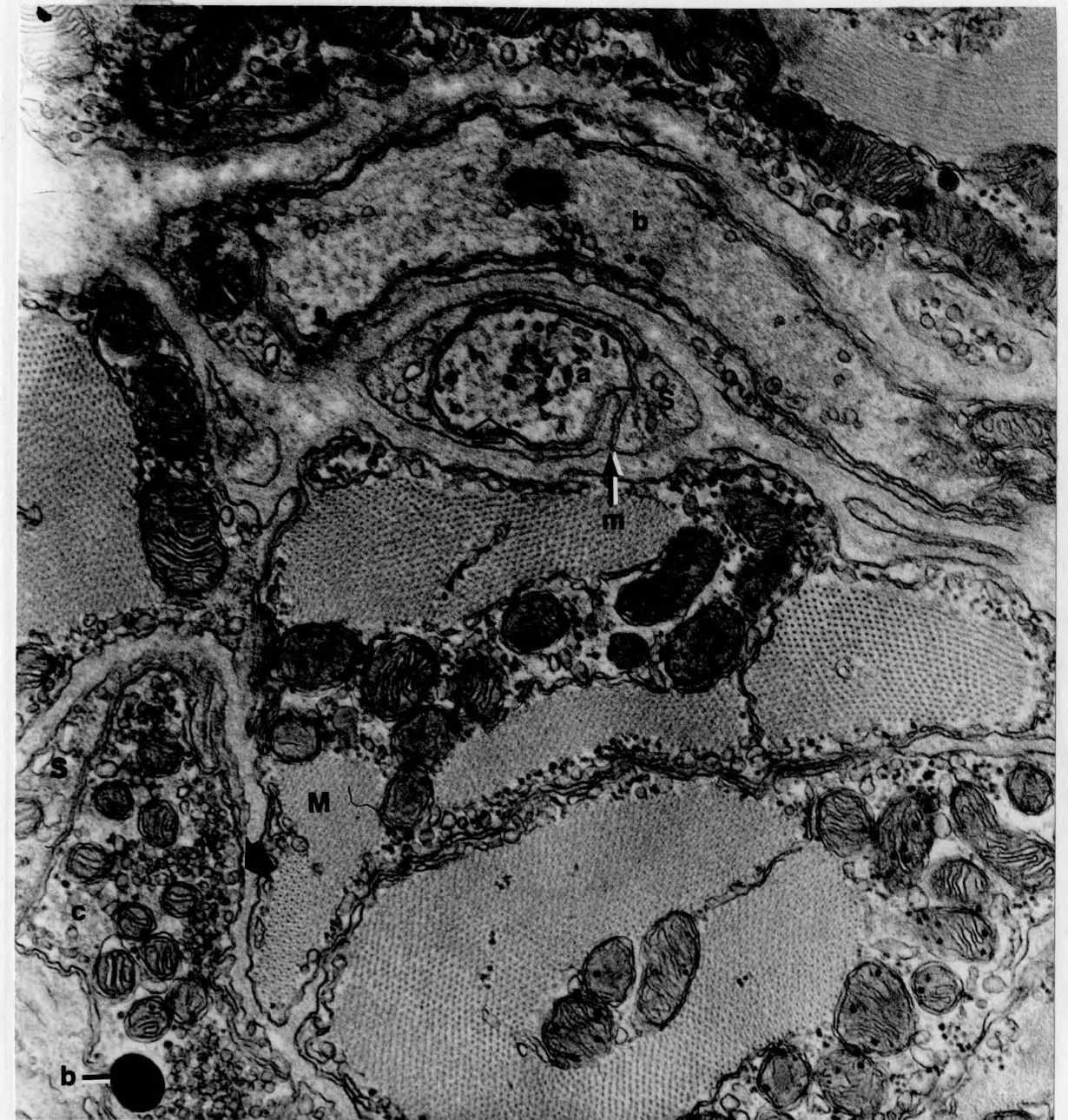


Figure 24

PLATE XIX

Figure 25:

Sinus venosus: In contrast to the axon "c" in Figure 24, the axoplasmic contents of these Schwann cell invested axons are characteristic of nerve not immediately associated with muscle cell membranes. Large dense core vesicles (empty arrow), mitochondria (m), microtubules (solid arrow), and neurofilaments (nf) are all in evidence including a multilaminated body (b). There is a conspicuous absence of electron lucent vesicles. 48,000X. OsO_4 , lead citrate. S, Schwann Cell.



Figure 25

PLATE XX

Figure 26:

Sinus venosus: This cross-sectional view of a large axon (A) shows mitochondria (m) oriented with the long axis of the axon. They are considerably smaller than the muscle cell mitochondria (M). Neurofilaments (nf) and microtubules comprise the other main axoplasmic contents. Golgi body (G) within the Schwann cell (S). 30,000X. OsO_4 , lead citrate.



Figure 26

PLATE XXI

Figure 27:

Neuromuscular association within sinus myocardium: The axon membrane is exposed (empty arrow) due to an incomplete Schwann cell investment (S) allowing it to come within 480A of the sarcolemma of an adjacent myocardial cell (M). The volume of the axon at this level is almost totally occupied by electron lucent vesicles (V) although three mitochondria are also present. Compared to the axon in Figure 26, axons involved in close neuromuscular relationships have a less evident number of microtubules and neurofilaments. 29,000X. OsO₄, lead citrate. S, Schwann cell.



Figure 27

PLATE XXII

Figure 28:

Sinus venosus: This section is a different level through the same axon shown in Figure 27. This section exhibits a slightly higher concentration of electron lucent vesicles (V) and a few more mitochondria. It has maintained its neuromuscular association with the muscle cell (M) shown in Figure 27 (empty arrow) but now another close association between an exposed portion of the same axon membrane and the sarcolemma of a second muscle cell (M) has been established (solid arrow). 34,000X. OsO₄, lead citrate.



Figure 28

PLATE XXIII

Figure 29:

Sinus venosus: This partially exposed axon protruding from its Schwann cell sheath (S) approaches to about 450A of the sarcolemma of an adjacent muscle cell (M) (empty arrow). Its axoplasmic contents include typical electron lucent vesicles (V) and numerous mitochondria. 30,000X. glutaraldehyde and OsO_4 , lead citrate.

Figure 30:

Sinus venosus: This axon is a different section of the one shown in Figure 29. An exposed portion of the axon is apposed (empty arrow) to the muscle cell (M). The axoplasm contains a few mitochondria and a reduced number of vesicles (v) with clear centers. The larger vesicles are approximately 450A in diameter and the narrowest distance between the axon membrane and the sarcolemma is about 500A. S, Schwann cell. 48,000X. glutaraldehyde and OsO_4 , lead citrate.

Figure 29

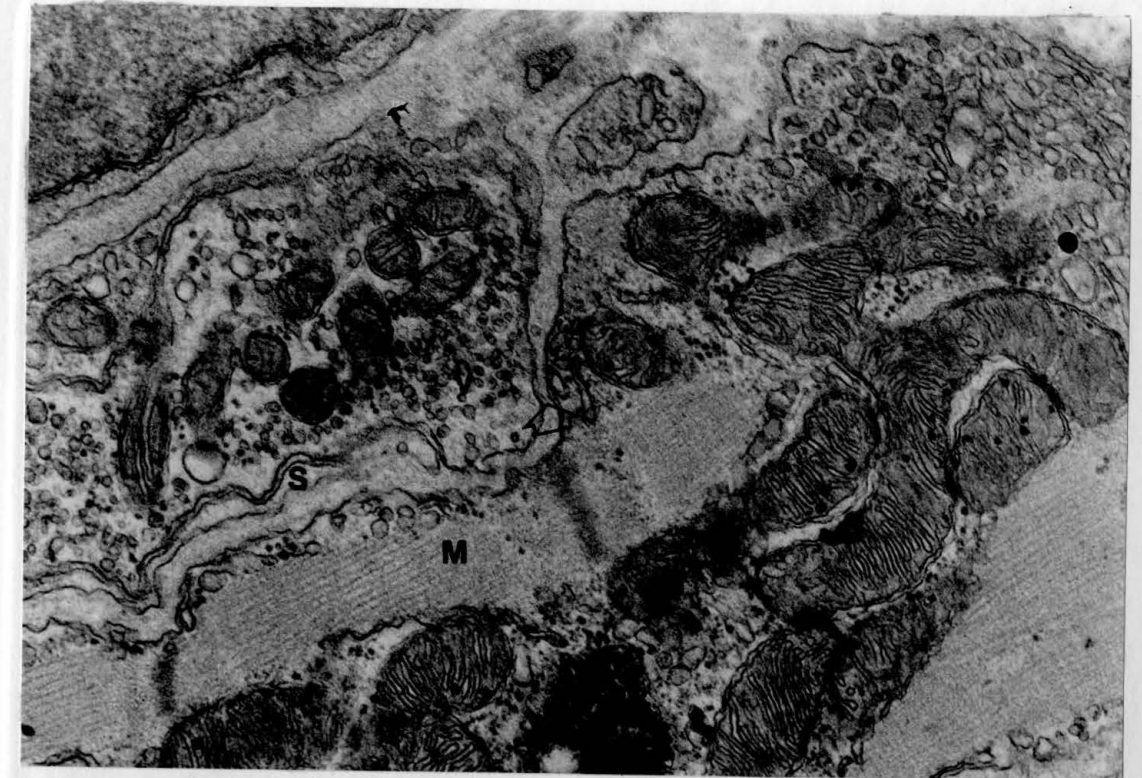


Figure 30

PLATE XXIV

Figure 31:

Sinus venosus: Tangential section through a neuromuscular association. A Schwann cell (S) partially encloses an axon (a) whose exposed face is directed toward a muscle cell (m). The basement membrane of the two cells intervene between them. The angle of section is known to be tangential due to the short "en face" view of the axon membrane (empty arrow). C, chromaffin cell. 44,000X. glutaraldehyde and OsO_4 , lead citrate. V, dispersed vesicles.

Figure 32:

Neuromuscular contiguity within sinus venosus: At one point, the bare axon approaches to within 150A (solid arrow) of the sarcolemma (s). The vesicles (V) within the axon have electron lucent cores. Immediately subjacent to the sarcolemma is a large subsarcolemmal cistern (C). 70,000X. glutaraldehyde, potassium dichromate, OsO_4 , lead citrate.

Figure 31

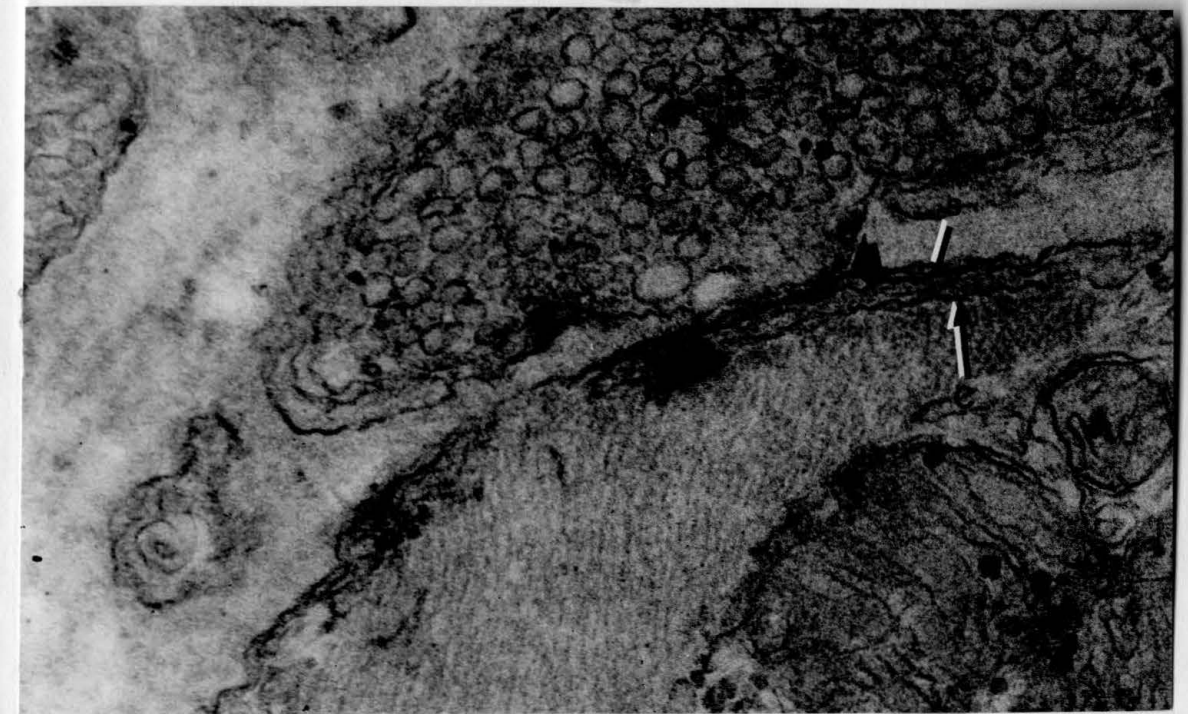


Figure 32

PLATE XXV

Figure 33:

Ventricle: Incubated in Karnovsky's acetylthiocholine medium. Note the presence of the electron dense reaction product on the endothelial lining (E), areas of the sarcolemma (S), and along the filaments, especially near the Z-line (Z) at the A-I junction (empty arrow). 30,000X. paraformaldehyde and OsO_4 , lead citrate

Figure 34:

Ventricle: Incubated in Karnovsky's butyrylthiocholine medium. The electron dense reaction product is distributed along the myofibrils, somewhat more heavily at the A-I junction (empty arrow). 41,000X. paraformaldehyde and OsO_4 , lead citrate. z, z-band.

Figure 33

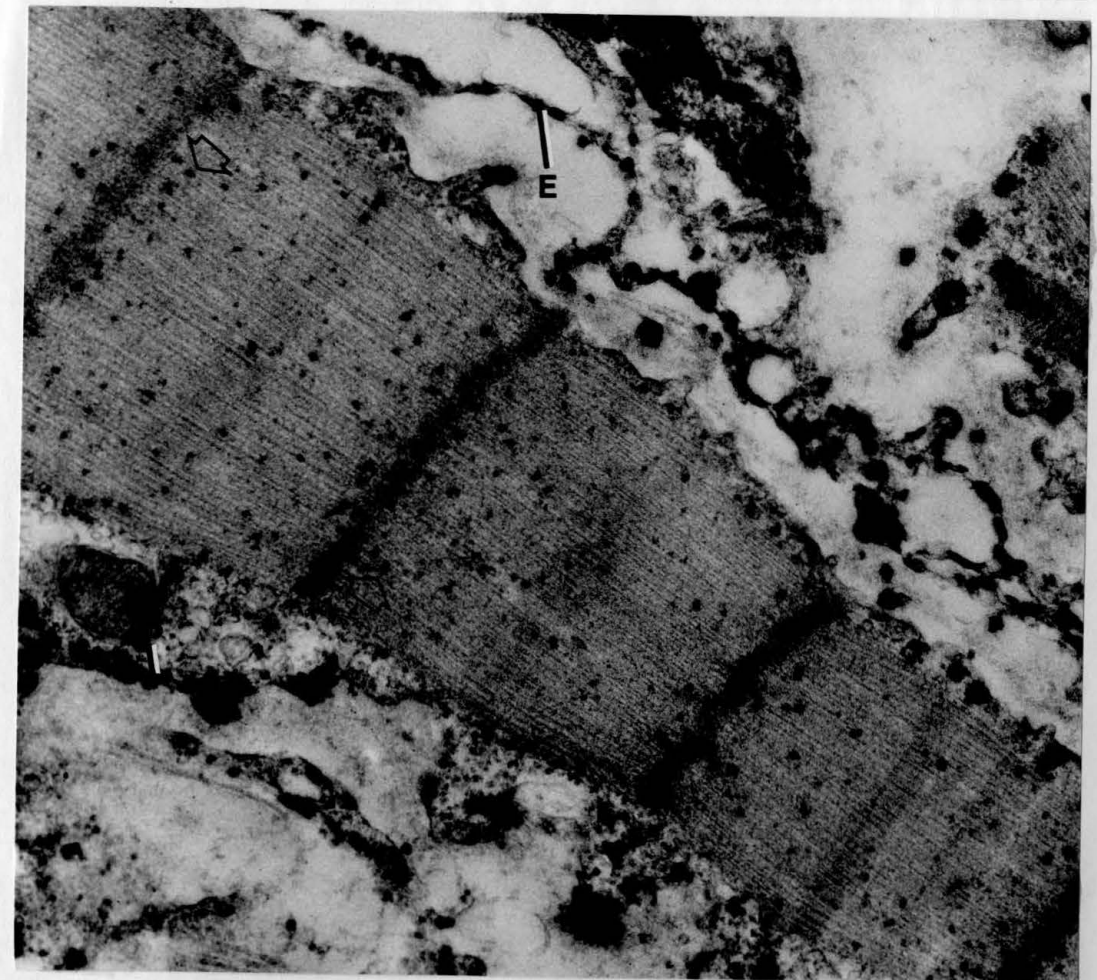


Figure 34

PLATE XXVI

Figure 35:

Ventricle: Two sarcomeres are shown with their constituent parts labelled. Z, Z-line; I, I-band; A, A-band; M, M-band. The empty arrow points to one of three cross-bridges resolvable within the M-band. 48,000X. glutaraldehyde and OsO_4 , lead citrate.

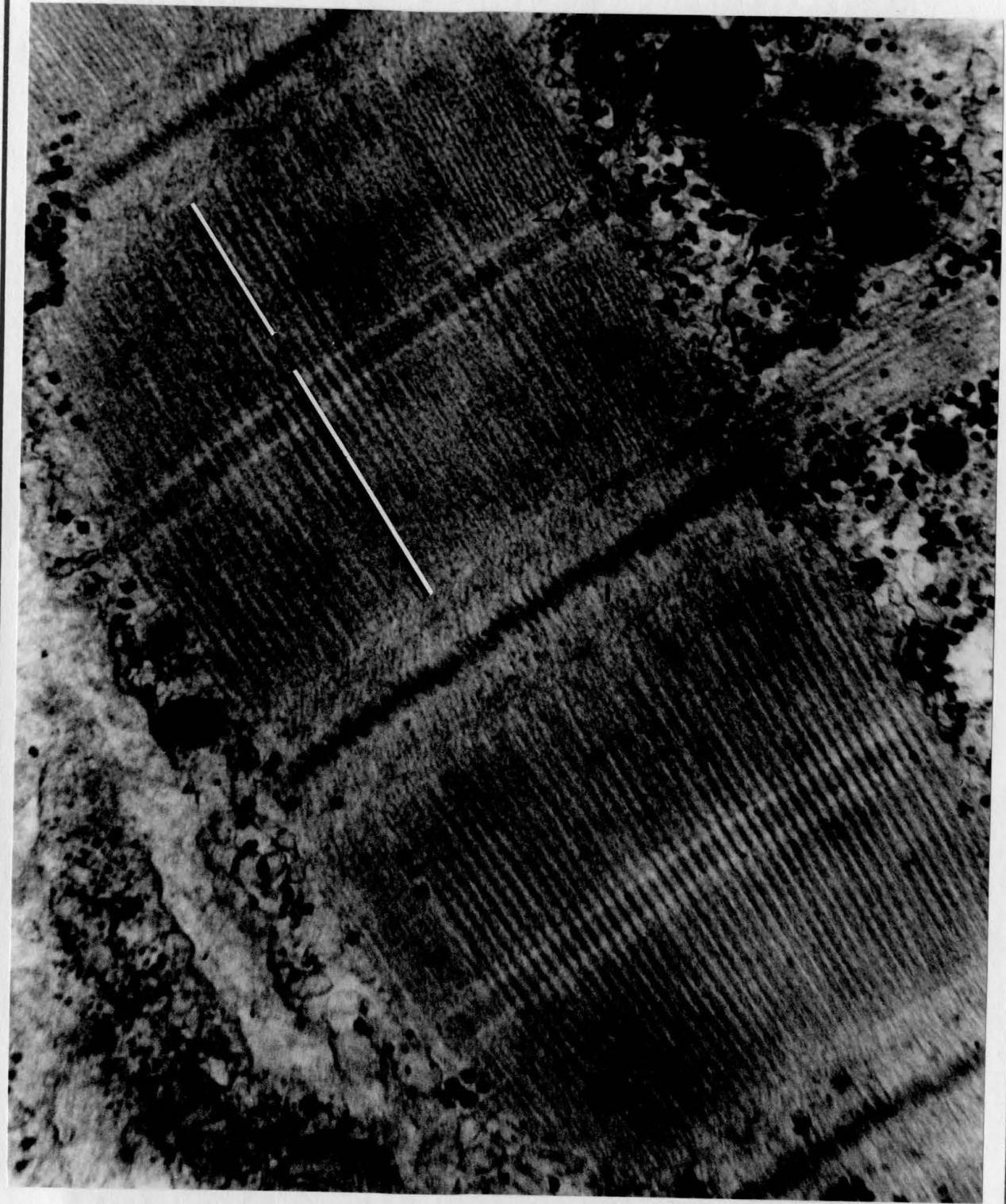


Figure 35

PLATE XXVII

Figure 36:

Ventricle: Incubated in Karnovsky's acetylthiocholine medium after pretreatment with N-ethylmaleimide. Electron dense reaction product is present within the subsarcolemmal cistern along the I-band (empty arrow). Stretches of the sarcoplasmic reticulum (SR) subjacent to the sarcolemma (S) but somewhat removed from the A-I junction are unreactive. Some electron dense reaction product is also present within the sarcoplasmic reticulum of another myocardial cell above (solid arrow). Since this section was not stained in lead citrate, the electron dense material cannot be attributable to lead precipitate. Note that the myofibrillar reaction product as observed in Figure 33 has been eliminated by the pretreatment with N-ethylmaleimide. 48,000X. paraformaldehyde and OsO_4 , unstained. z, z-band.

Figure 37:

Ventricle: Incubated in Karnovsky's acetylthiocholine medium after pretreatment with N-ethylmaleimide. Segments of sarcoplasmic reticulum (solid arrow) subjacent to the sarcolemma are unreactive although a nominal amount of reactivity is in evidence along part of the sarcoplasmic reticulum of a neighboring muscle cell (empty arrow). No myofibrillar activity is present. Z, z-band; I, I-band. 63,000X. paraformaldehyde and OsO_4 , lead citrate.

Figure 36

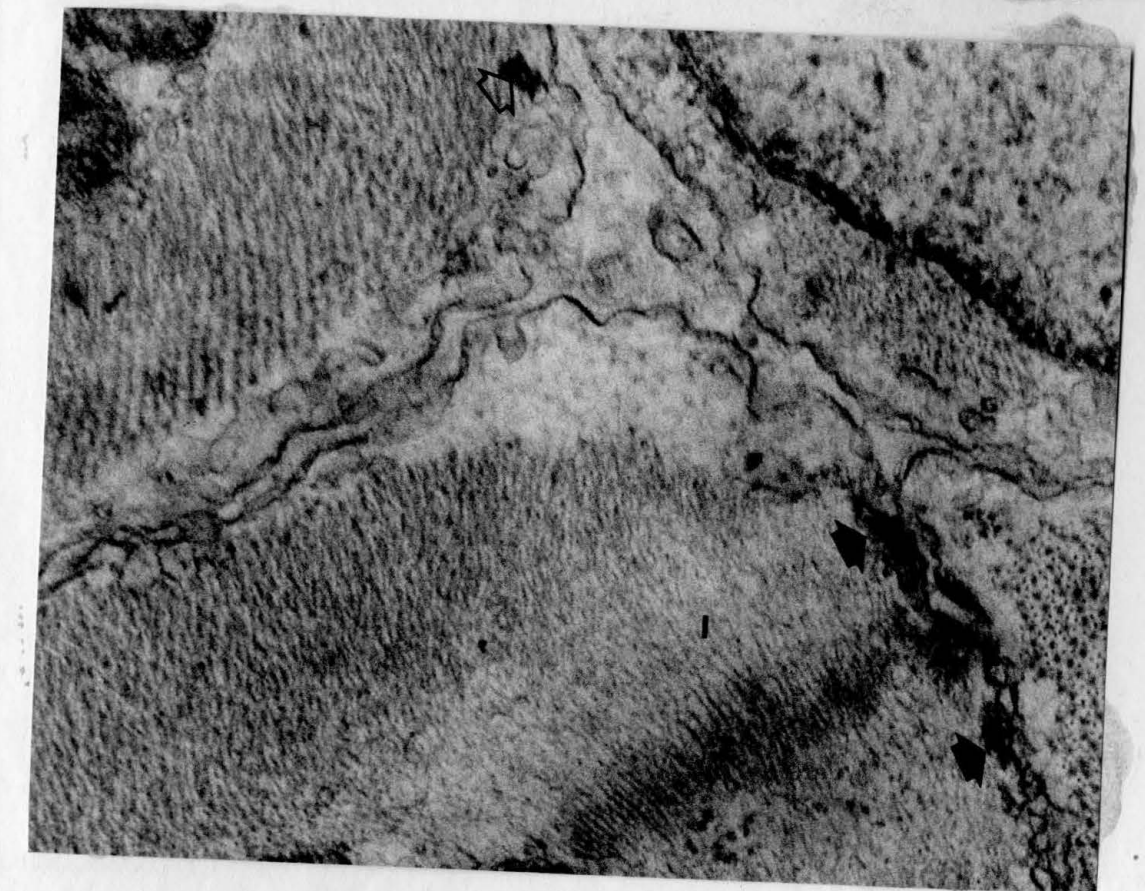


Figure 37

PLATE XXVIII

Figure 38:

Ventricle: Incubated in Karnovsky's acetylthiocholine medium after pretreatment with N-ethylmaleimide. Electron dense reaction product is present within the sarcoplasmic reticulum (arrows) of two apposing muscle cells. The reactive membranes are situated along the I-band. No reaction of myofibrillae has occurred. Z, z-band; A, A-band; I, I-band; S, sarcolemma. 66,240X. paraformaldehyde and OsO_4 , lead citrate.

Figure 39:

Ventricle: Incubated in Karnovsky's medium without substrate after pretreatment with N-ethylmaleimide. There is no evidence of reaction product within the sarcoplasmic reticulum (arrows) under the sarcolemmae of two neighboring cells. Z, z-band. 48,000X. paraformaldehyde and OsO_4 , lead citrate.

Figure 38

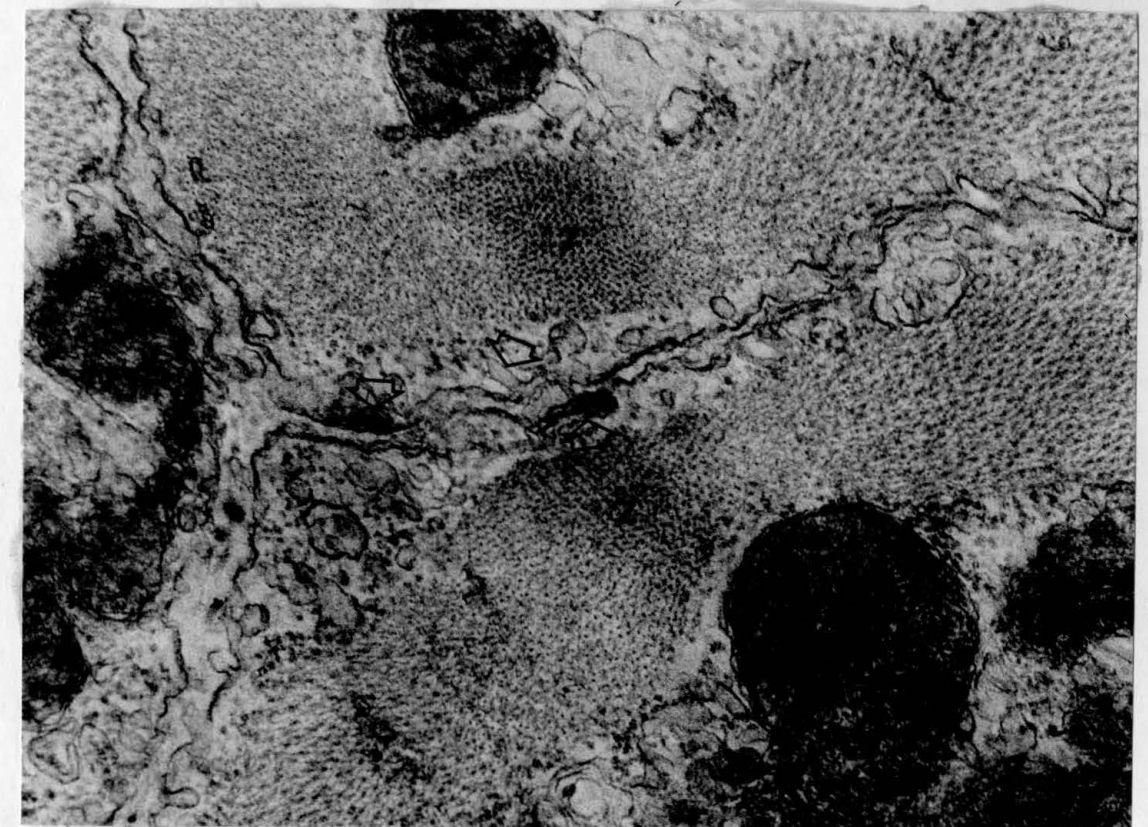
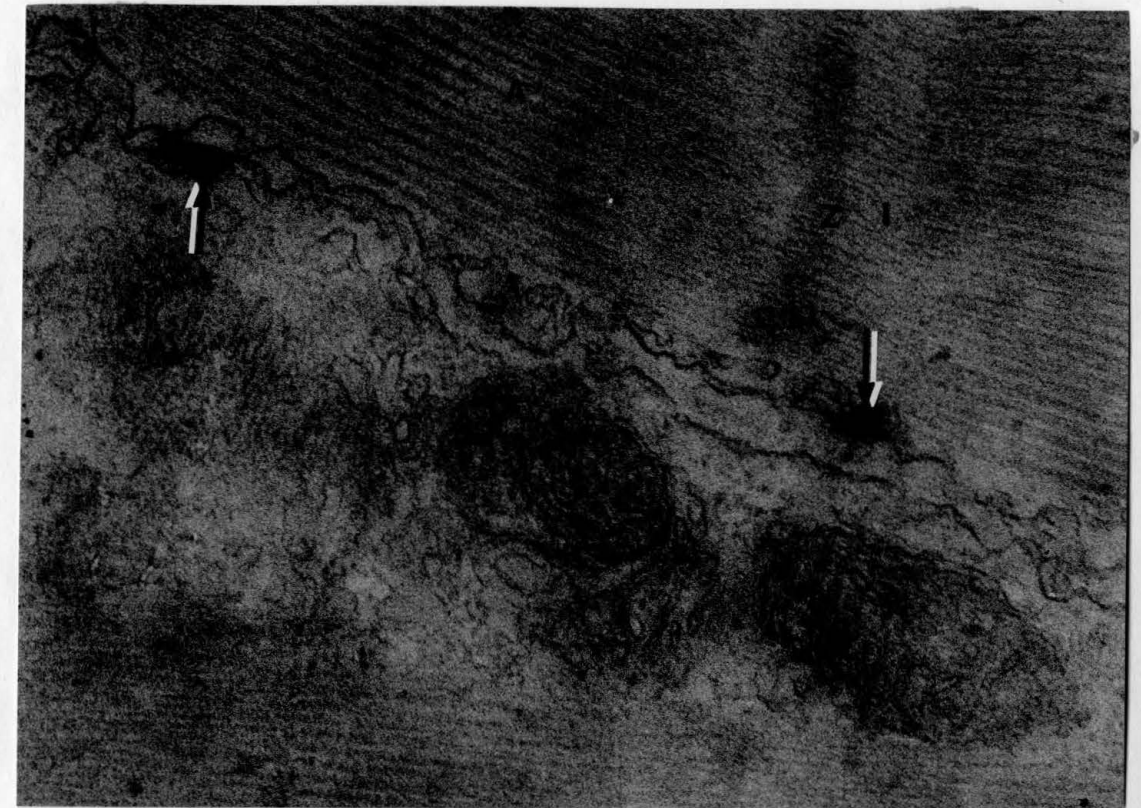


Figure 39

PLATE XXIX

Figure 40:

Ventricle: Incubated in Karnovsky's acetylthiocholine medium with tetraisopropylpyrophosphoramidate after pre-treatment with N-ethylmaleimide. Arrow points to reactive sarcoplasmic reticulum. See the enlargement of this area in Figure 41. Z, z-band; S, sarcolemma; E, endothelial lining. 52,000X. paraformaldehyde and OsO_4 , lead citrate.

Figure 41:

Area of reaction product from Figure 40 enlarged to 162,000X. Note that the reaction product has deposited in focal points along the membrane of the sarcoplasmic reticulum (arrow). These reactive centers are spaced about 150A apart. S, sarcolemma; Z, z-band. paraformaldehyde and OsO_4 , lead citrate.

Figure 40

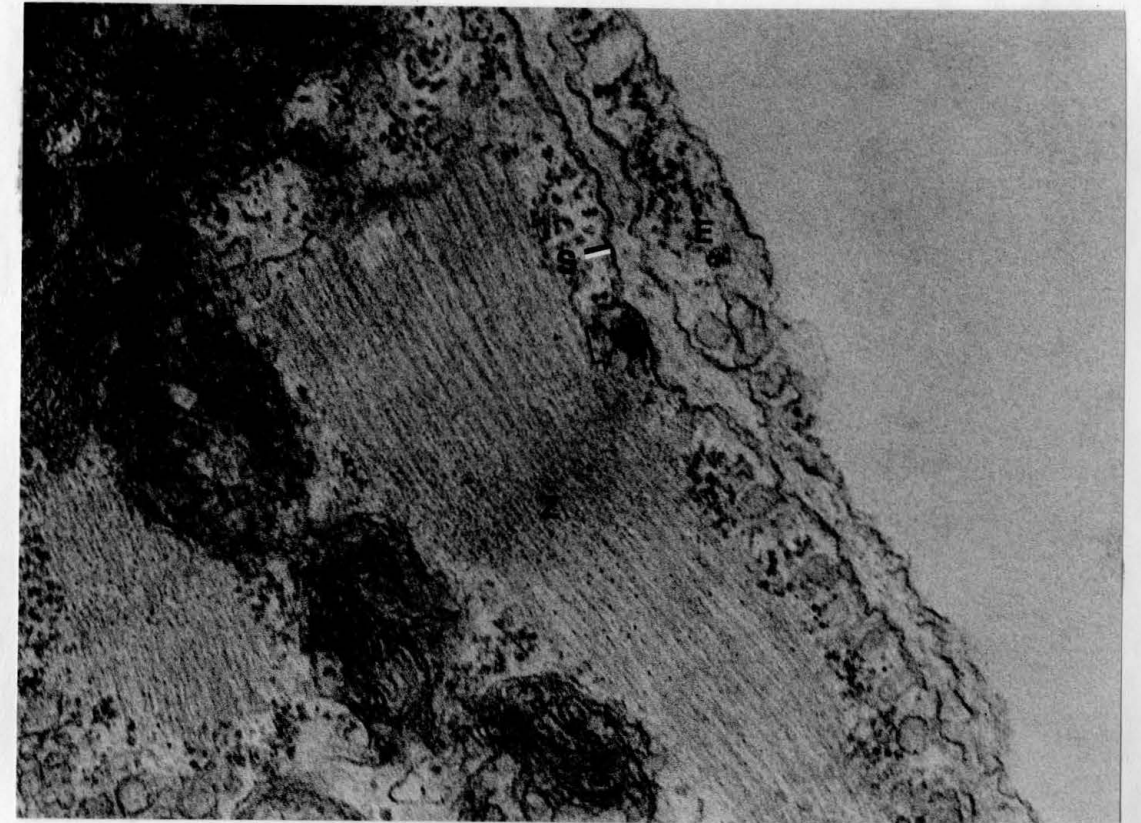


Figure 41

PLATE XXX

Figure 42:

Ventricle: Incubated in Karnovsky's acetylthiocholine medium with eserine sulfate (a group specific anticholinesterase) after pretreatment with N-ethylmaleimide. Arrows point to unreactive sarcoplasmic reticulum. A, A-band; Z, z-band. 64,500X. paraformaldehyde and OsO_4 , lead citrate.

Figure 43:

Ventricle: Incubated in Karnovsky's acetylthiocholine medium with B.W.284C.51 (a competitive inhibitor of specific acetylcholinesterase) after pretreatment with N-ethylmaleimide. Arrows indicate unreactive segments of sarcoplasmic reticulum subjacent to the sarcolemma. A, A-band; Z, z-line; I, I-band; S, sarcolemma. 45,000X. paraformaldehyde and OsO_4 , lead citrate.

Figure 42

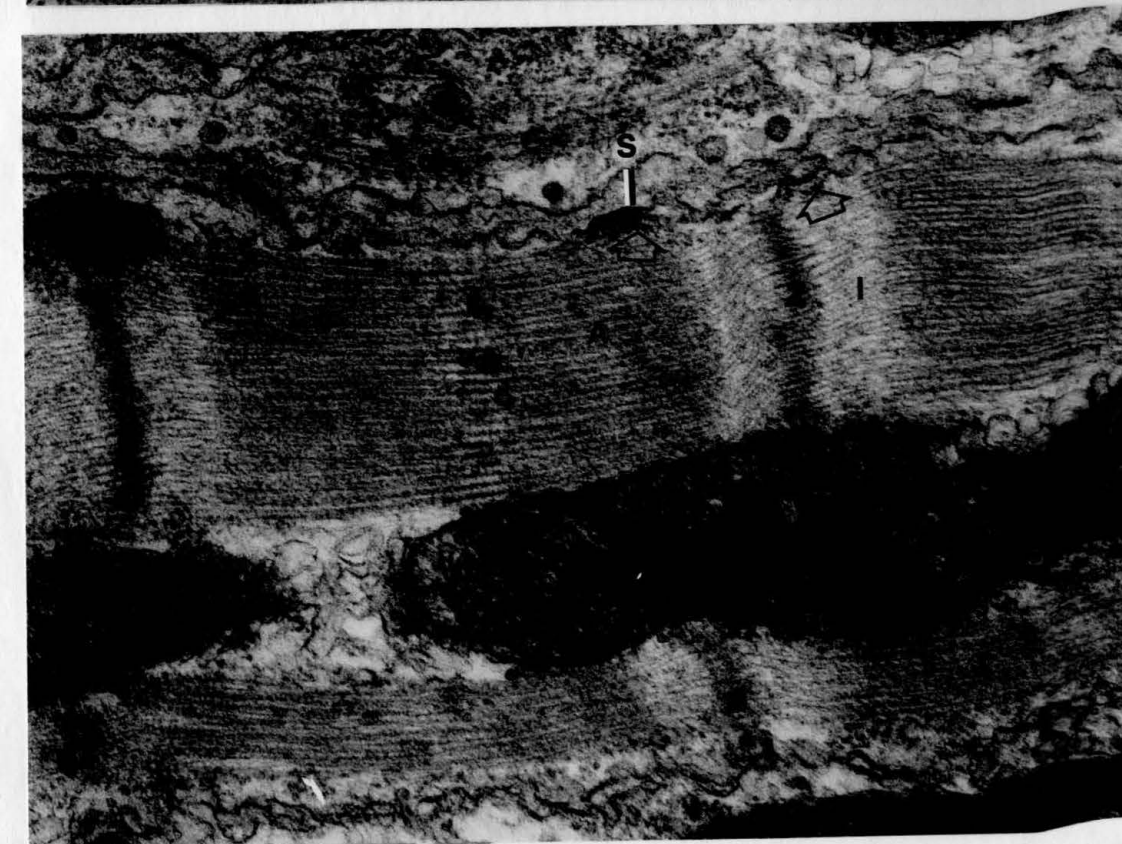
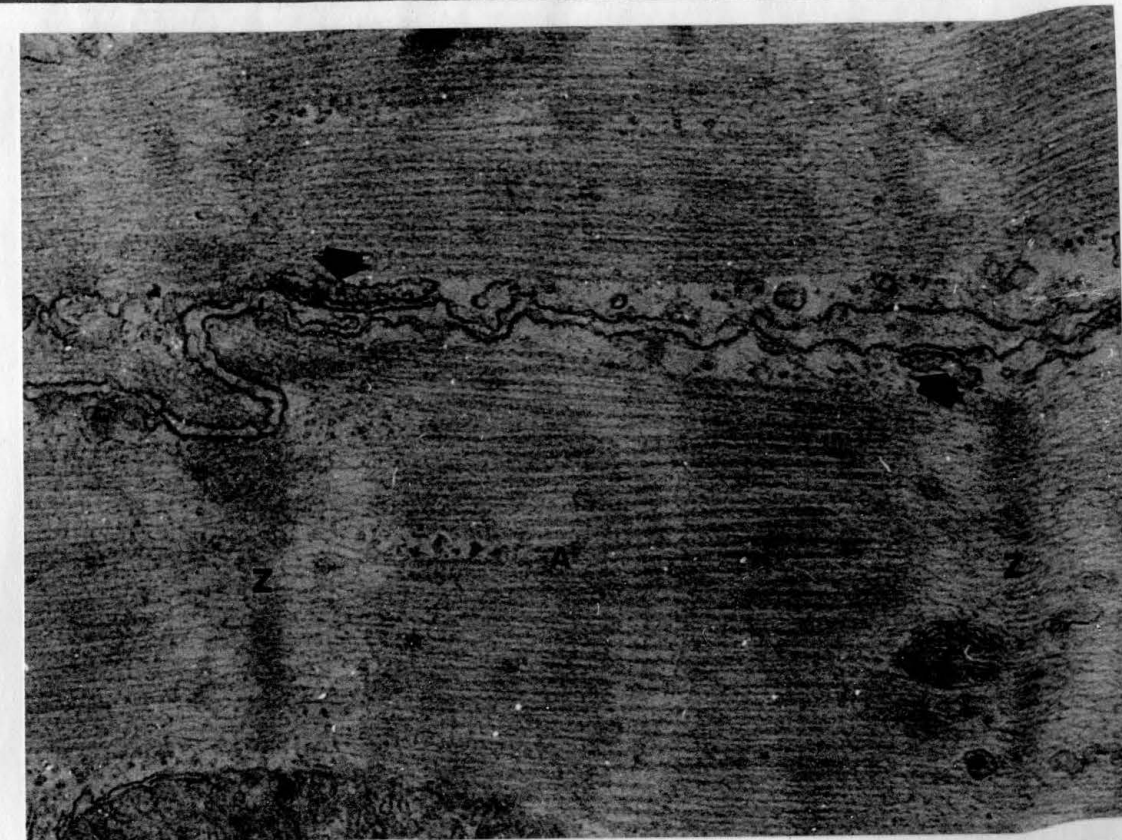


Figure 43

PLATE XXXI

Figure 44:

Ventricle: Solid arrow points out a subsarcolemmal cistern with a linear electron dense material in its center. The empty arrow points to another subsarcolemmal cistern without such an organized central density. Both types of membranes span between the z-line (Z) and the edge of the A-band (A). It is believed that the acetylcholinesterase activity demonstrated in Figures 36, 38 and 40 is attributable to these portions of the sarcoplasmic reticulum. 20,350X. glutaraldehyde and OsO_4 . uranyl acetate and lead citrate.

Figure 45:

Area from Figure 44 enlarged to 43,450X. The solid arrow indicates the subsarcolemmal cistern with the central density and the empty arrow points to the lighter staining subsarcolemmal cistern without a central density. glutaraldehyde and OsO_4 . uranyl acetate and lead citrate.

Figure 46:

Ventricle (inset): Solid arrow shows a subsarcolemmal cistern with the electron-dense center. The empty arrow shows that this SSC is continuous with sarcoplasmic reticulum without a central density. 56,250X. glutaraldehyde and OsO_4 . uranyl acetate and lead citrate. Z, z-band.

Figure 44

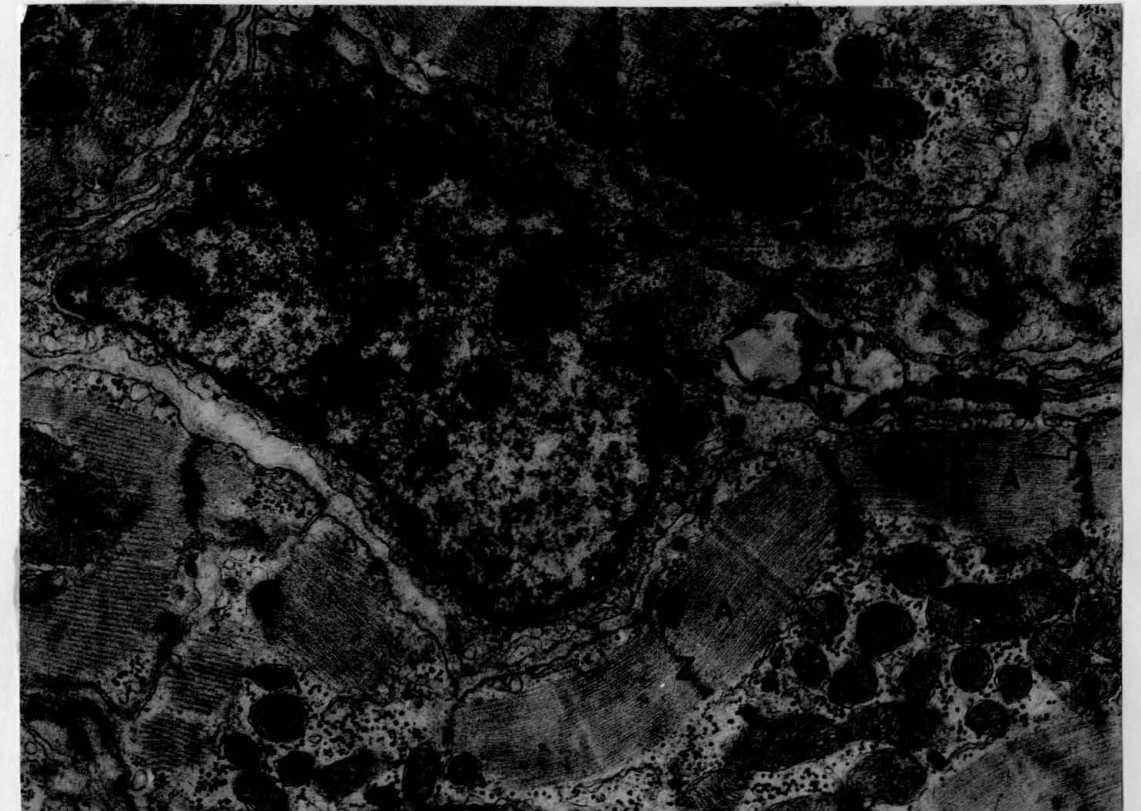


Figure 45

Figure 46

PLATE XXXII

Figure 47:

Ventricular myocardial cell (M): Subsarcolemmal cisternae near the surface of the myocardial cell are indicated by empty arrows. Solid arrows point to underlying sarcoplasmic reticulum with which the subsarcolemmal cisternae are continuous. m, myofibrils; G, membrane bound electron dense granules. 48,000X. glutaraldehyde and OsO_4 . uranyl acetate and lead citrate.



Figure 47

APPROVAL SHEET

The dissertation submitted by Theodore M. Beringer has been read and approved by members of the faculty of the Graduate School of Loyola University of Chicago.

The final copies have been examined by the chairman of the thesis committee and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given final approval with reference to content, form and accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

01/07/72

Date

Robert Hasler

Signature of Advisor